Enantioseparation using a counter-current bioreactor

A thesis submitted for the degree of Doctor of Philosophy by

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Abbreviations and Nomenclature

abs	Absorbance
ACN	Acetonitrile
ATPS	Aqueous two-phase system
BF_4	Tetrafluoroborate
BIB	Brunel Institute for Bioengineering
BMIM	1-butyl-3-methylimidazolium
BSA	Bovine serum albumin
CCC	Countercurrent chromatography
CCS	Centrifugal contactor-separator
CPC	Centrifugal partition chromatography
CS	Chiral selector
CV	Coefficient of variation
D	Distribution ratio
Da	Dalton
DAAO	D-amino acid oxidase
DCM	Dichloromethane
DE	Dynamic Extractions Limited
DOM	Dextrorphan
DOMe	Dextromethorphan
E. coli	*Escherichia coli*
ee	Enantiomeric excess
EMIM	1-ethyl-3-methylimidazolium
EtOAC	Ethyl acetate
F	Mobile phase flow rate (ml/min)
H	Head
HILIC	Hydrophilic interaction chromatography column
HPLC	High performance liquid chromatography
HSCCC	High speed countercurrent chromatography
IEX	Ion-exchange chromatography
IL	Ionic liquid
KBA	Keto butyric acid
K_D	Partition ratio
K_m	Michaelis-Menten constant (mg)
LC	Liquid chromatography
LP	Lower phase
MAO	Mono amino acid oxidase
MDM	Multiple Dual-Mode
MeOH	Methanol
MP	Mobile phase
mPEG-Mal	Methoxy-PEG-Maleimide
M_r	Relative molecular mass (g/mol)
$N(CN)_2$	Dicyanamide
NTf_2	Bis(trifluoromethylsulfonyl)imide
OD	Optical density (AU)
OPA	*ortho*-phthalaldehyde
$PC_6C_6C_6C_{14}$	Trihexyltetradecylphosphonium

PEG	Polyethylene glycol
PEG-MS	Polyethylene Glycol Monostearate
PF_6	Hexafluorophosphate
PTFE	Polytetrafluoroethylene
RP	Reversed phase
rpm	Revolutions per minute
R_T	Retention time (min)
SD	Standard deviation
Sf	Stationary phase retention (ml)
SP	Stationary phase
T	Tail
tBME	Methyl *tert*-butyl ether
TvDAAO	*Trigonopsis variabilis* D-amino acid oxidase
UP	Upper phase
UV	Ultra violet
v	Volume (ml)
V_{max}	Maximum rate of the enzymatic reaction
W	Weight (mg)
λ	Wavelength

Abstract

The potential of countercurrent chromatography (CCC) as a small footprint bioreactor/separator for manufacture of enantiopure chiral molecules was explored, using as a model reaction the isolation of L-amino butyric acid (L-ABA) from a DL-ABA racemate and the enantioselectivity of D-amino acid oxidase (DAAO). Bioconversion of D-ABA to ketobutyric acid (KBA) by DAAO, immobilised by selective partitioning in the stationary phase of the CCC centrifuge, was accompanied by separation of unreacted L-ABA from KBA by the countercurrent action of the centrifuge.

For effective bioreactor/separator action, a high partition of the biocatalyst to the stationary phase was required in order to retain the biocatalyst in the coil, with differing partitions of substrates and products between the stationary phase (SP) and mobile phase (MP) so that these could be separated. Aqueous two-phase systems (ATPS) were the major two-phase systems used to provide SP and MP, as these are well reported to be effective in preserving enzyme activity. The distribution ratios of DL-ABA, KBA and DAAO were measured in a range of phases with polyethylene glycols (PEGs) of different molecular weights, different salts, and different compositions of PEG and salt, using an automated robotic method, developed for the purpose. A system of 14% w/w PEG 1000/ 14% w/w potassium phosphate, pH 7.6, gave the best combination of distributions ratios ($C_{PEG\ phase}/C_{salt\ phase} = C_{SP}/C_{MP}$) for ABA, KBA and biocatalyst (DAAO) of 0.6, 2.4 and 19.6 respectively. A limited number of aqueous-organic and ionic liquid two-phase systems were also reviewed, but found unsatisfactory.

CCC operating conditions such as substrate concentration, biocatalyst concentration, the mobile phase flow rate (residence time in the CCC coil), temperature, rotational speed and operational modes (single flow and multiple-dual flow) and types of mixing (cascade and wave-like) were optimised to produce total conversion of D-ABA to KBA, which was then completely separated from unreacted, enantiomerically pure (>99% ee), L-ABA.

Advantages of the CCC bioreactor over conventional technology include reduced equipment footprint, cheaper running costs, and faster purifications. However, in its current format the drawbacks, such as enzyme instability and excessive optimisation time, reduce its commercial appeal. Additional investigations into the use of whole cell preparations of biocatalyst in the CCC bioreactor showed potential to overcome the problem of enzyme instability and this may in the future give the CCC bioreactor a place in the enantioseparation field.

Acknowledgments

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1. INTRODUCTION AND LITERATURE REVIEW

1.1. Aims and Objectives

The aims and objectives of the research project presented in this thesis are listed bellow:

1) Development of analytical procedures

To allow the enzyme activity as well as partitioning of amino butyric acid (ABA), ketobutyric acid (KBA) and enzyme in a range of phase systems, to be determined analytically.

2) Phase system selection

Identification and optimisation of a two-phase system able to maintain the enzyme active enough to complete the conversion of injected substrate and allow unaltered L-ABA and KBA produced within the CCC-coil to elute at a different time.

3) Optimisation of the CCC-bioreactor

To obtain a very high D-ABA degradation within the CCC coil, which was necessary to attain L-ABA of 99% enantiomeric purity.

4) Purification of enantiomerically pure L-ABA from a racemic mixture

To allow isolation of unaltered L-ABA, which is the target compound, from KBA produced within the CCC column.

Overall, the aim of the research project presented in this thesis was creating a bioreactor based on a CCC centrifuge, to enable the enantioseparation of L-amino acid of at least 99% enantiomeric purity from the racemic mixture of DL-aminobutyric acid.

1.2. Introduction to chromatography

Chromatography is a separation technique thought to be invented in 1906 by the Russian scientist Mikhail Semyonovich Tsvet, who applied it to the separation of plant pigments during his research on chlorophyll using columns of calcium carbonate. The term *chromatography* comes from Greek χρώμα: *chroma*, colour and γραφειν: *graphein*, to write. However, a number of commentators have pointed that Tsvet named the technique after himself, literally "Tsvet's writing", as the word *tsvet* in the Russian language means colour.[1]

1.2.1. Chromatographic theory

Chromatography is the collective term for a set of techniques used for the separation of mixtures. The technique, which involves passing a mixture of different molecules, dissolved in the mobile phase, through a stationary phase is based on the differential partitioning between these two phases. Molecules which interact with the stationary phase need more time to pass through, therefore they elute at different times.

The aim of all chromatography is the separation, or resolution, of compounds in the mixture. The quality of resolution is defined by the resolution factor (R_S) determined according to equation 1. R_S is expressed as the ratio of the distance between the two peak maxima (d) to the mean peak width (W) at the baseline.[2]

R_S higher than 1.5 means that the two peaks are separated with the baseline return, whereas a resolution factor lower than 1.5 means that there is some peak overlap.[2]

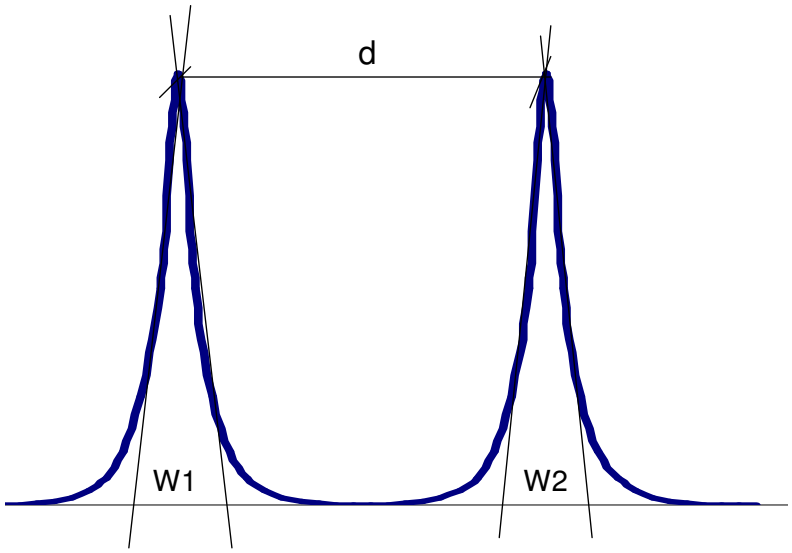


Figure 1 Resolution between a two peaks. d – the distance between the two peak maxima; W1 and W2 – width of the two peaks at the baseline

$$Rs = \frac{d}{(W_1 + W_2)/2} \qquad \textit{Equation } 1$$

1.2.2. Review of chromatographic techniques

The various chromatographic techniques are named according to the state of the mobile phase. Liquid chromatography (LC) is a technique where the mobile phase is a liquid, whereas gas chromatography (GC) uses a gas as the mobile phase. A further subclassification was made according to the state of the stationary phase e.g. column or planar chromatography.

Planar chromatography is a separation technique where the stationary phase is present on a plane, such as paper or a glass plate covered by a layer of the stationary phase. An example of planar chromatography is thin layer chromatography (TLC).
In the case of column chromatography the stationary bed is located within a tube (column). Since the mobile phase can be either a liquid or a gas, column chromatography can be divided into LC and GC.

1.2.2.1. Gas chromatography (GC)

Gas chromatography (GC) is that form of chromatography in which a gas is the mobile phase. The sample is vaporized and carried by the gaseous, mobile phase (*the carrier gas*). Samples partition into the stationary phase, which usually is a liquid,

based on their solubilities at a given temperature. The components of the sample separate from one another based on their relative vapour pressure and affinities to the stationary phase.[3]

Although GC is a fast, efficient and sensitive technique, the method has some disadvantages such as limitation to volatile samples. Samples containing high concentrations of nonvolatile molecules, such as salts or PEG, cannot be analysed directly as those molecules, unless removed before an injection, would build up in the coil. For that reason GC is not convenient for analysing samples of an aqueous two phase system (ATPS).

1.2.2.2. Liquid Chromatography (LC)

Liquid chromatography (LC) is a separation technique which uses a liquid mobile phase and can be carried out either in a column or on a plane. The stationary phase applied for that technique can be either a solid or a liquid.

The most common LC technique is high-performance liquid chromatography (or high pressure liquid chromatography, HPLC). It is a very efficient method to separate, identify and quantify different molecules. The stationary phase might contain various functional groups such as $C_{18}H_{37}$ attached to a solid support which usually is silica beads, packed in a column. The size of beads is in the order of 5μm, which creates a very high pressure inside the column, up to 6000psi. The smaller the beads the higher the resolution therefore, for modern HPLC, columns are filled with particles that are smaller than 2μm. This generates a pressure reaching 15,000psi, which requires special pumps. This type of HPLC system is called Ultra High Pressure Liquid Chromatography (UHPLC).

Depending on the functional groups attached to the silica beads, different types of columns are available for use in HPLC:

- **Normal Phase HPLC (NP-HPLC)** was one of the first types of HPLC used for separation of polar compounds. The column is typically filled with silica beads which intrinsically are very polar. The mobile phase consists of a very nonpolar solvent, like hexane or heptane, mixed with a slightly more polar solvent like isopropanol, ethyl acetate or chloroform. Recently its modification, HILIC bonded phases are becoming popular.

- **Hydrophilic Interaction Chromatography (HILIC)** is a version of normal phase liquid chromatography. The polar stationary phase is usually made of silica beads modified by attaching polar groups such as amino, amide, cationic or zwitterionic groups. The separation is achieved by hydrophilic partitioning mechanisms between the mobile phase and the water-enriched layer in the hydrophilic stationary phase, superimposed on weak electrostatic interactions between charged analytes and the neutral zwitterionic stationary phase (Figure 2). That results in a unique selectivity, which is especially suitable for analytes that are poorly retained on reversed phase columns.

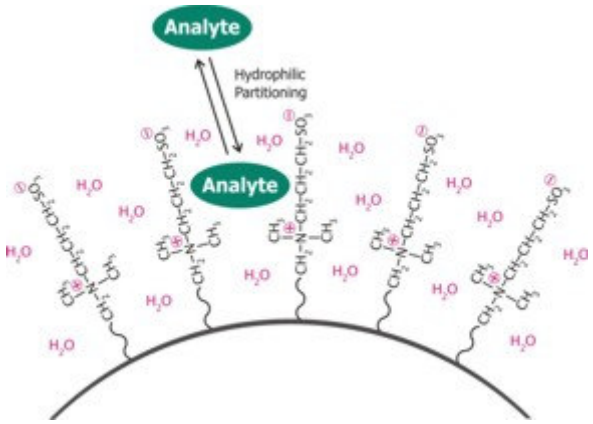


Figure 2 Separation process for the ZIC-HILIC column made by SeQuant. Taken from www.sequant.com

- **Reversed Phase HPLC (RP-HPLC)** uses moderately polar mobile phase and a non-polar stationary phase, which is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17} attached to silica beads. The method is used for separation of moderately nonpolar molecules, which are very common compounds in nature. Reversed phase chromatography operates on the principle of hydrophobic forces, therefore the less polar the compound, the higher the retention on the column. To decrease retention a less polar mobile phase is used, usually by adding methanol or acetonitrile. Frequently a gradient elution is applied by gradually increasing the concentration of less polar solvent in the mobile phase. This accelerates the elution of highly nonpolar compounds from the column.

- **Ion-exchange chromatography (IEX)** allows separation of ions and charged molecules, such as amino acids, based on their charge. The stationary phase surface displays ionic functional groups, which interact with an analyte of opposite charge. This type of chromatography is further subdivided into cation exchange chromatography and anion exchange chromatography.
- **Size exclusion chromatography** is a technique which separates molecules based on their size. Small molecules, trapped in pores of the stationary phase particles, need more time to elute than big molecules. Those which are too big to enter the pores simply pass through the column. Because of very low resolution, the technique is often used as a final step of purification of macromolecules, such as proteins and polymers.
- **Affinity chromatography** is a method of separating biochemical mixtures based on highly specific biological interactions, such as that between a protein and an antibody, enzyme–substrate or receptor-ligand. The separation procedure usually consists of two steps. First a mixture is loaded onto the equilibrated column. The compound of interest is temporarily bound to the column, whereas the flow-through containing impurities is discarded. The next step involves flushing the column with a wash solvent, which washes out the previously bound compound of interest.

1.2.3. Analytical and preparative chromatography

Analytical chromatography, which usually requires a small amount of a sample, is used for the identification and quantification of molecules. However, chromatography can only be used for the identification of already defined molecules by comparison of elution time for eluted compounds and their standards.

The most widely used chromatography techniques for analytical purposes are HPLC and GC. In both cases the mobile phase coming out from a column passes through a detector, which emits a signal proportional to the concentration of eluent passing at any time through the detector. The signal, which is recorded by a computer, is plotted against the elution time. The area under a peak is proportional to the concentration of the eluted compound.

Preparative chromatography is used for isolating compounds from a mixture of chemicals. Fractions of mobile phase eluted from a column are collected such that each compound of interest is present in a different set of fractions. Examples of preparative chromatography are IEX, affinity chromatography, HPLC and CCC.

1.3. Countercurrent chromatography (CCC)

Countercurrent chromatography is a liquid-liquid separation technique without any solid support invented by Ito in late 1960s[4]. In CCC both the mobile phase as well as the stationary phase are liquid. To retain the liquid-stationary phase in a column, a centrifugal force is used, while the second immiscible phase is passed through as a stream making contact with the stationary phase. The separation is based upon the partitioning of an analyte between the stationary and the mobile phase.

The technique has several advantages over commonly used techniques such as preparative HPLC. Since both phases are liquid, a sample can always be recovered, either from blowing out the coil contents or applying elution-extrusion[5]. Therefore, unlike in HPLC, the irreversible adsorption or loss of a sample in the column cannot occur. A very high percent of the column (up to 70-90%) is a liquid stationary phase. That allows for injecting as much sample as 10-30% of the column volume. Since both phases are liquid, either can be used as mobile or they can even be switched over during a run.

The technique has been used to separate natural products[6], synthetic molecules[7], proteins[8], organelles[9], cells[10, 11] and bacteria[12].

1.3.1. Types of phase-mixing in CCC

In order to improve the mixing and separation of the two phases, a number of different mixing modes have been developed. The two main mixing modes are hydrodynamic and hydrostatic mixing.[13]

- In hydrodynamic CCC a wave of mixing and settling of the two phases passes through the column. Therefore, if the flow stops, the phases will redistributed according to the rule described in section 1.3.2, i.e. the heavy phase gathers in the tail end, whereas the light phase tends to the head end of a coil. Since the coil of a hydrodynamic CCC consists of a tube wound around a bobbin rotating in the planetary motion, no sealing system is required and the process is not pressure

limited. A coil made of PTFE tube is also easy to clean. Moreover, the process can be easily scaled up[14,15] by applying a large bore coil such as 17.5mm, allowing throughput in excess of 25kg/day[16]. The disadvantage of this type of column comes from its sensitivity to changes in interfacial tension between the phases, so it does not retain an aqueous two-phase system (ATPS) well.

- In hydrostatic CCC the stationary phase is trapped in a series of chambers, thus if the flow stops it will remain there. In spite of some advantages, such as retaining "difficult" phase systems for instance, ATPS provides much more efficiency in comparison to hydrodynamic CCC, and better mixing between the phases, this type of column also has some limitations. The main disadvantage is related to the limited length of a column due to the increasing pressure. Moreover, a system of chambers is more difficult to clean compared with the tube used in hydrodynamic CCC. Hydrostatic CCC, in comparison to hydrodynamic CCC, is also more difficult to scale-up or scale-down.

1.3.2. Principle of hydrodynamic countercurrent chromatography

The liquid stationary phase is retained in the coil by the Archimedean screw force, which is generated by rotating the coil. Figure 3 shows that, in a slowly rotating coil filled with water, both air bubbles and metal beads migrate to the same end of the coil, called the head of a coil. The opposite end of the coil is called the tail.[17]

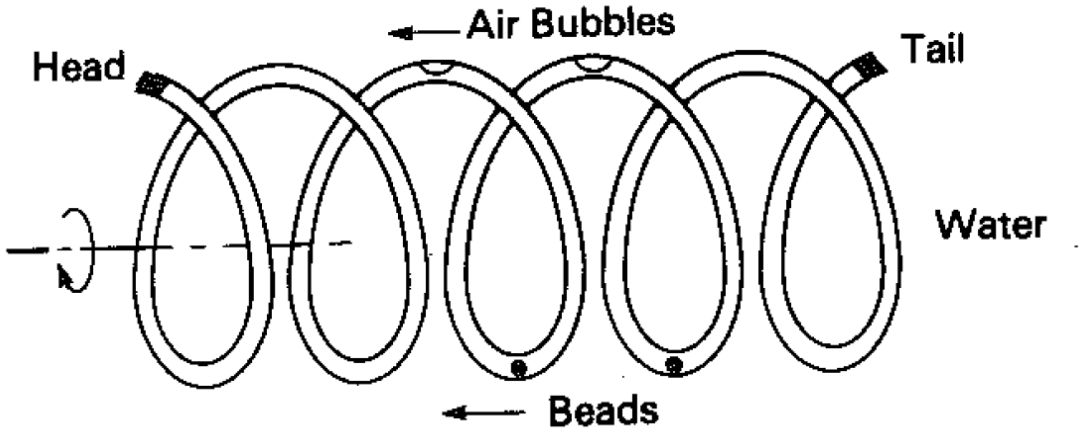


Figure 3 Motion of various objects in a slowly rotating coil. Inside the helically wound coil filled with water there are air bubbles and beads. If the coil is slowly rotated around its helical axis, air bubbles as well as beads tend to move towards one end of the coil, contractually named the head of the coil. The other end is called the tail. (Taken from Ito and Conway (1996)[17]).

If the coil filled with two immiscible phases is rotated, both phases tend to move towards the head of the coil. Since they cannot occupy the same place, the lighter-upper phase occupies the head of the coil whereas the lower phase is displaced towards the tail. Therefore, to maintain the stationary phase in a coil, if the lower phase is the mobile phase it should be pumped from head to tail, and the upper phase in the opposite direction (tail to head) if it is used as the mobile phase.

If a spinning coil is entirely filled with one phase (i.e. the stationary phase, SP) and the other phase flowed over it (i.e. the mobile phase, MP), some of the SP is going to be displaced till the hydrodynamic equilibrium is obtained. When a mixture of different compounds, having different partition coefficients between the stationary and the mobile phase, is injected onto the coil, they will elute from the other end of the coil at a time governed by their partitioning (K_D) between the phases. If the analyte is only soluble in the mobile phase ($K_D = 0$), it will elute with the solvent front of the mobile phase. Alternatively it will stay in the column if it is soluble only in the stationary phase ($K_D = \infty$). However, since the stationary phase can be pumped out from the column, a totally retained analyte can easily be recovered.

1.3.3. Distribution ratio (D)

The retention time of an injected analyte in CCC depends on its affinity for the stationary and the mobile phase. Since both the mobile as well as the stationary phase are liquid, partitioning of an analyte can easy be determined by analysing its concentration in those phases.

The distribution ratio (D) is the total analytical concentration of an analyte in each liquid phase of the two phase system, regardless of a chemical form of the analyte.

The distribution constant or partition ratio (K_D) is the ratio of the concentration of an analyte in a single form between the two phases. In countercurrent chromatography it is the ratio of a concentration of an analyte in the stationary phase $[A]_{stat.}$ and the mobile phase $[A]_{mobile}$ at equilibrium:

$$K_D = \frac{[A]_{stat.}}{[A]_{mobile}} \qquad \textit{Equation } 2$$

Often there is no chemical reaction, ionisation or complexation in the two liquid phases, in which case D = K_D.

K_D values of many solutes are directly analysed by the so-called *shake-flask* method. The solute is partitioned between the two liquid phases of a proposed solvent system in a tube. After equilibrium is achieved the concentration of the analyte in each phase is determined using a variety of techniques such as HPLC, GC or biological assays.[18]

These values show the affinity of an analyte to both phases, thus they determine the time an injected analyte elutes from a CCC coil (retention time, R_T). In countercurrent chromatography the retention time of an analyte can be predicted according to equation 3:

$$R_T = \frac{K_D \cdot V_S + V_M + V_{extra}}{F} \text{; [min]} \qquad \textit{Equation } 3$$

K_D - distribution constant, V_S – stationary phase volume [ml], V_M – mobile phase volume [ml], V_{extra} - extra coil volume [ml], F – mobile phase flow rate [ml/min]

Values V_S and V_M can be calculated based on the volume of the stationary phase displaced (V_{disp}) to equilibrate the coil of the volume V_C [ml].

$$V_C = V_S + V_M \qquad \textit{Equation } 4$$

$$V_C = V_{disp} + V_S - V_{extra} \qquad \textit{Equation } 5$$

1.3.4. CCC setup

Figure 4 shows a typical CCC setup used for the separation of compounds. Two pumps supply the CCC centrifuge with the mobile and the stationary phase. Before the CCC, there is an injection port used for injecting a sample.

The injection port is based on a 6-way selection valve operated in the two modes: load or injection. In the load position the sample loop, which is made of a certain length of a PTFE or stainless steel tube, is filled with a sample. When the valve is set

in the inject position, the stream of mobile phase passing through the sample loop injects its content onto the coil.

The mobile phase coming out from a CCC-centrifuge can pass into a detector, connected to a computer, which records signals as chromatograms. Since CCC is a preparative technique, almost always fractions of the eluted mobile phase are collected using a fraction collector. For blowing out phases from the CCC-centrifuge, to extrude compounds which do not elute during a CCC run, compressed nitrogen is often used.

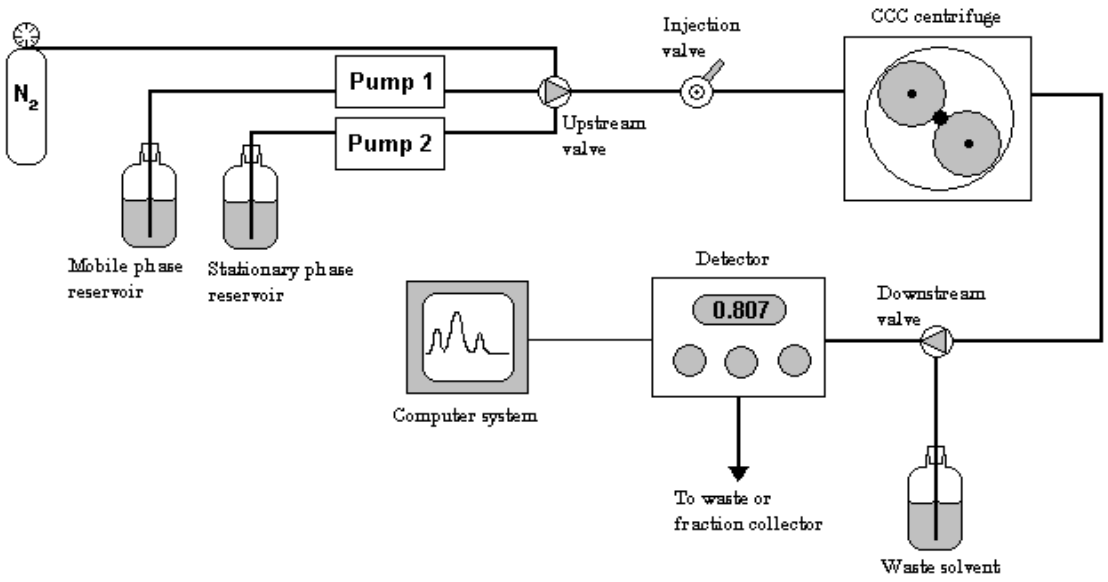


Figure 4. Schematic diagram of a typical CCC set up taken from Garrard *et al* (2008)[19]

1.3.4.1. Hydrodynamic CCC

The tubing is wound on a bobbin which rotates on a planetary axis (Figure 5) in such way that at the proximal (nearest) side of the planetary coil there is mixing between two immiscible liquid phases in the tube, whereas at the distal (furthest) side there is settling between the two phases. Rotating the coil creates a wave of simultaneous mixing and settling zones which travel synchronously towards the head end of the tubing. A sample injected on a coil would experience 1000 mixing and settling zones a minute when the main rotor speed is 1000rpm.[20]

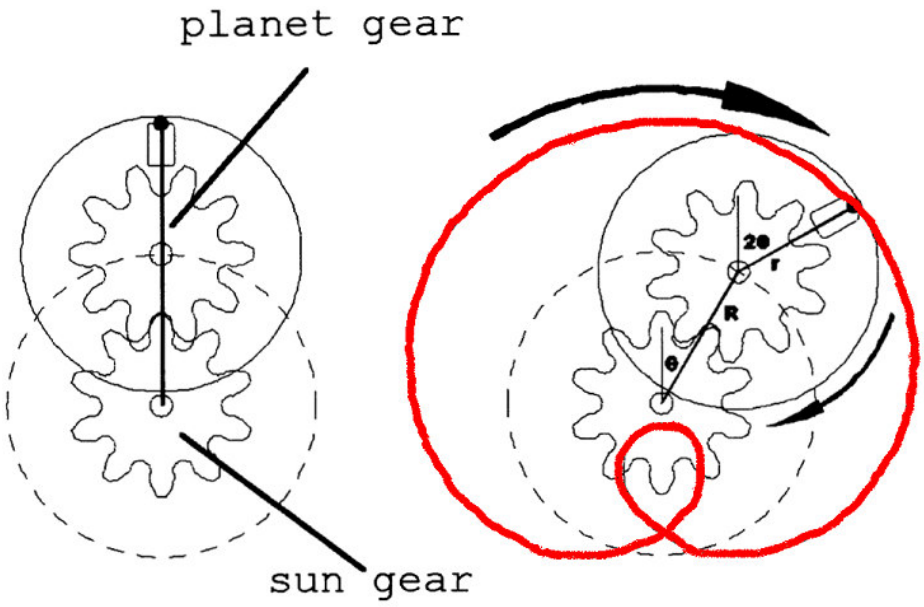


Figure 5 Planetary motion of CCC column. The red line indicates the path traced by a point of the coil.

The planetary motion is created by mounting the planetary gear on the coil holder axis to an identical sun gear rigidly fixed to the centrifuge framework.[21] The column rotates around its own axis while revolving around the centrifuge axis, like the rotating earth revolves around the sun. Both the rotation and the revolution are at the same angular velocity (ω) and in the same direction (synchronous).

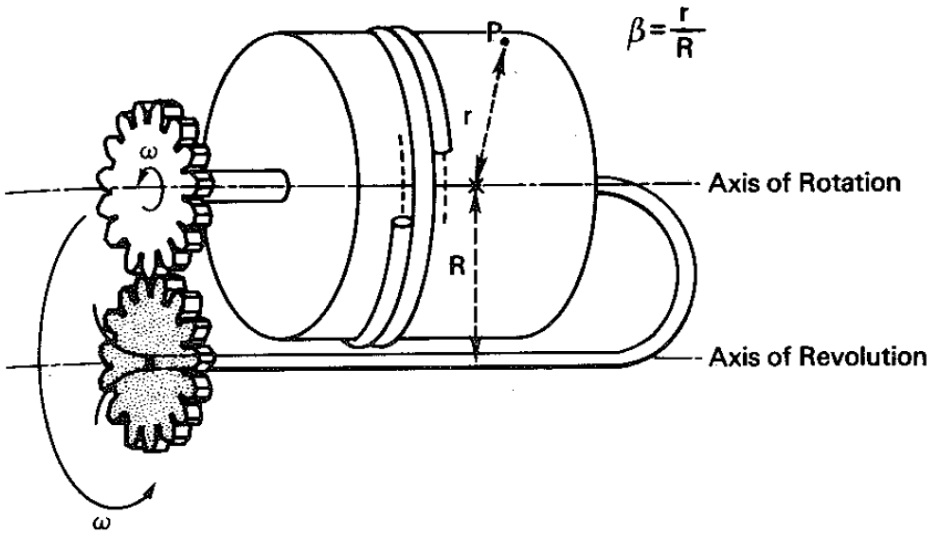


Figure 6 Design principle of the J-Type CCC. The bobbin rotates around its own axis and revolves around the centrifuge axis at the same angular velocity (ω) in the same direction (synchronous motion). That rotation prevents twisting of the flow leads, which eliminates the need for seals. Taken from Ito and Conway (1996)[17]

In planetary coil rotation there are two zones where different forces and processes take place. In the peripheral part of the coil from the axis of revolution, there are centrifugal forces which cause separation of the two phases, whereas, the proximal part of the rotating coil makes a loop which causes mixing of the two phases as shown in Figure 7 based on a spiral coil. When the molecules migrate with the mobile phase through the rotating coil, they pass alternately zones of mixing and zones of separation.

The other consequence of synchronous moving is that the flying leads do not twist. Therefore no rotating seals are required, which eliminates the risk of a phase leaking or contamination.

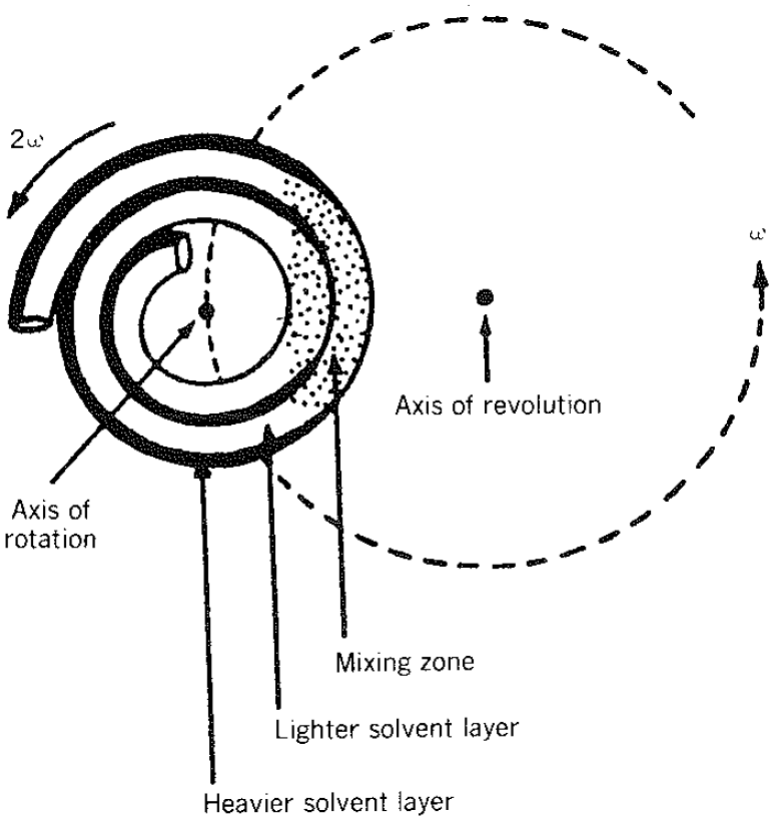


Figure 7 Solvent distribution in a multilayer coil. Taken from Ito and Conway (1996)[17]

Many coils are made by winding a tube in several layers. In a multilayer coil there are at least two forces that influence the two immiscible phases:

1. The Archimedean screw action forces the heavy phase to the tail
2. Hydrostatic pressure forces the heavy phase to the periphery of a multilayer coil

The best stationary phase retention is obtained when the two forces are coupled rather then competing. Therefore, it is desirable to have the tail of the coil at the periphery

and the head at the centre. Table 1 shows the orientation of the head and the tail of a coil according to winding and spinning direction. For the best retention, a clockwise wound coil ought to be rotated in the clockwise direction, whereas a counter clockwise wound coil in counter clockwise direction.

	Counterclockwise Coil		**Clockwise coil**	
Spun Clockwise	Periphery	Head	Periphery	Tail
	Centre	Tail	Centre	Head
Spun Counterclockwise	Periphery	Tail	Periphery	Head
	Centre	Head	Centre	Tail

Table 1 Orientation of the Head and the Tail for a coil wound clockwise and counter clockwise, spun clockwise or counter clockwise.

The mobile phase can be pumped in two directions: from the head to the tail and from the tail to the head. The heavier phase tends to occupy the tail of a coil. If that phase is chosen as the stationary phase, the solvent inlet must be at the tail of the column. Therefore, the mobile, upper phase should be pumped from the tail (periphery) of the coil to its head (centre). Table 2 shows the pumping direction of the mobile phase, depending on whether the upper or the lower phase is used.

Mobile phase	**Direction of the mobile phase pumping**
Upper phase	from the tail (periphery) to the head (centre)
Lower phase	from the head (centre) to the tail (periphery)

Table 2 Direction of the mobile phase pumping.

Different CCC operating modes

- **Isocratic Mode**. The composition of the mobile phase is the same through the whole separation process.
  - **Normal Phase Mode** is when the stationary phase is the polar one and the mobile phase is nonpolar one. For most aqueous-organic phase systems the polar phase is the lower, aqueous one. It is recommended to pump the upper, mobile phase from Tail (Periphery) to Head (Centre).
  - **Reverse Phase Mode** is when the stationary phase is the nonpolar one and the mobile the polar one. It is recommended to pump the upper mobile phase from Head (Centre) to Tail (Periphery).
- **Gradient elution.** The composition of the mobile phase changes during a separation.
- **Co-current chromatography.** Both phases are pumped in the same direction at different flow rates. This operating mode allows an analyte having a very high affinity to the stationary phase to elute.[22]
- **Dual Mode.** The mobile phase is initially pumped in the recommended direction e.g. from tail to head if it is the lower phase. Then both the mobile phase and the flow direction are altered. Thus, the stationary phase becomes the mobile phase and would be pumped from head to tail.
- **Continuous counter-current extraction.** Both phases are mobile and are pumped at the same time in opposite directions to each other. The sample is supplied continuously to the centre of the column. This mode requires a special design of CCC column.[23, 24]
- **Intermittent counter-current extraction.** This uses a series of alternating flow, between normal phase operation and reversed phase operation with the sample continuously injected in the middle.[25]

1.3.4.2. Hydrostatic CCC (CPC)

In hydrostatic CCC, when the flow rate stops the distribution of the stationary phase does not change when the centrifugal field is maintained. An example of hydrostatic CCC is centrifugal partition chromatography (CPC), where the stationary phase is held in cells or compartments under the action of a uniform field and the mobile phase flows through these compartments serially.

A CPC column consists of a number of chambers serially connected by ducts, placed on the rotor of a centrifuge (Figure 8). The stationary phase is maintained in chambers by a constant centrifugal force field produced by rotation around a single axis.
The mobile phase can be delivered to the ducts in two ways:

- descending (i.e. with the centrifugal force)
- ascending (i.e. against the centrifugal force)

When the mobile phase is the lighter of the two phases, (the upper-phase) it is pumped in ascending mode, whereas the heavier, lower phase is pumped in descending mode.

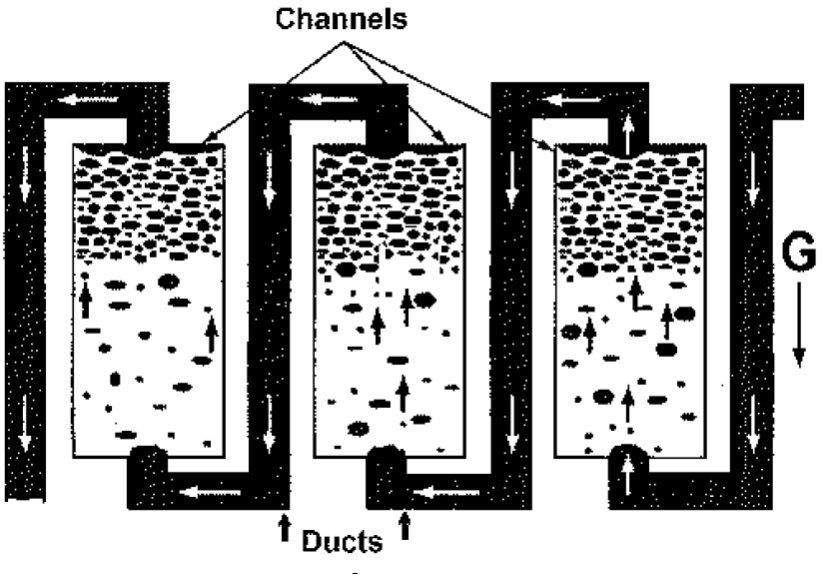


Figure 8. A schematic visualisation of chambers and ducts that make a CPC column.

It has been found that hydrostatic CCC is more efficient in retaining ATPS for example PEG1000/ potassium phosphate[26].

1.3.4.3. Toroidal CCC (TCCC)

A toroidal coil is a helically wound tube, either mounted circumferentially around a rotating disc[27] or wound on a rotating, planetary motion, large diameter, cylindrical coil holder[28] (Figure 9).

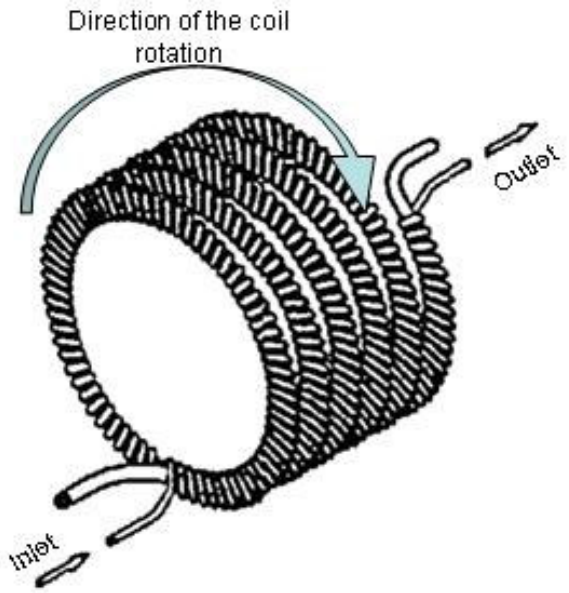


Figure 9 Toroidal coil wound around a rotating bobbin

A toroidally-wound coil, which is a pseudo-hydrostatic CCC, matches features of both hydrostatic and hydrodynamic CCC. In a similar manner to hydrostatic CCC, a toroidal coil provides vigorous, cascade mixing between the stationary and the mobile phase, enabling effective mass transfer which results in a good resolution the between separated compounds. That is especially beneficial if phases of high viscosity, like ATPS, are used. Analogously to a hydrodynamic CCC, a toroidal coil is wound of either PTFE or a stainless steel tube and therefore much easier to clean compared to a hydrostatic CCC coil made of inter-connected chambers. Moreover a toroidal type coil can be easily scaled-up by increasing an internal diameter of a tube.

Rotating a toroidal coil in synchronous planetary motion (Figure 10) like J-type CCC, allows continuous elution without using rotating seals[28].

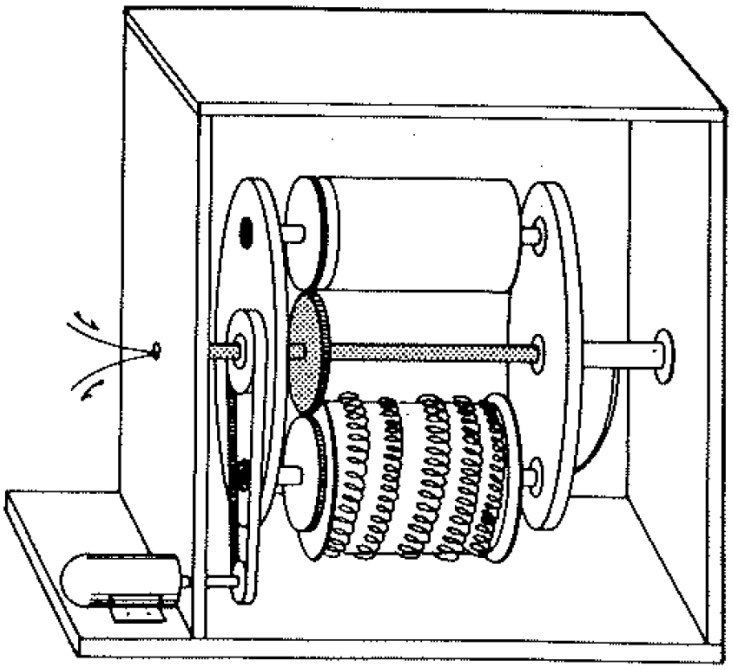


Figure 10 Overall view on the toroidal coil planet centrifuge. (From Ref. 28)

Using two model proteins, myoglobin and lysozyme, and ATPS composed of PEG1000 and K_2HPO_4 as a phase system, Guan *et al*[29] made a direct comparison between a toroidal coil and hydrodynamic J-type centrifuge. Due to inefficient mass transfer between the two phases, the J-type centrifuge of 45.1ml coil volume did not provide any separation of the two proteins. By contrast, the proteins were partly separated when a toroidal coil of volume 51.7ml was used and totally resolved when the coil volume was doubled (107.1ml) by doubling the coil length. Moreover when the toroidal coil was used, no stripping of the stationary phase (i.e. elution of the stationary phase from the column) was noticed as has often been observed in the hydrostatic CCC (CPC)[30].

Shinomiya *et al*[31] have also demonstrated a complete separation of the two model proteins, myoglobin and lysozyme, on a toroidal coil using ATPS composed of PEG1000 and K_2HPO_4 as a phase system.

1.3.5. Types of Two-Phase System for CCC

Phase system selection[32] plays the key role in the CCC separation as the technique is based on the differential partitioning between two immiscible phases (section 1.3.3).

There is a whole range of available phase systems that can be applied to CCC and searching for the most suitable two-phase system may be estimated as 90% of the entire work in CCC. Selecting the most appropriate solvent system frequently starts with a literature review of the separation of similar compounds in CCC.

The choice of solvent system is determined by the nature of separated molecules and criteria such as the polarity of the analyte and its solubility. Net charge as well as stability and ability to create complexes should also be taken into consideration.

In the next sections two-phase systems based on organic solvents, polyethylene glycol and ionic liquids are described.

1.3.5.1. Aqueous-organic phase systems

A wide spectrum of available organic solvents enables a wide range of different combinations and proportions. This results in a whole range of potentially useful phase systems for countercurrent chromatography, which might make the selection of a suitable phase system a very complicated and laborious procedure. Therefore, some efforts were put into classifying organic solvent systems according to their polarity. Abbott and Kleiman[33] (1991) classified solvent systems into three groups: lipophilic, polar and intermediate, based on a polarity determined using Reichardt's dye, which absorbs light in solution between 400 and 900nm depending on the polarity of the solvent.

Aqueous organic two-phase systems are the most commonly used solvent systems in countercurrent chromatography. Since they are mixtures of water and various organic solvents, their low viscosity and relatively low density makes these phase systems convenient in operating CCC. Moreover the high volatility of most organic solvents enables an easy isolation of a fractionated analyte from the phase system by evaporation.

1.3.5.2. Aqueous two-phase system (ATPS)

Aqueous two-phase systems are composed of water and either two immiscible polymers or a polymer and a salt, which above a critical concentration makes two immiscible phases.

In 1896 a Dutch microbiologist, Martinus Beijerinck, noticed the immiscibility of aqueous solutions of gelatine and an agar or starch, which mixed together were creating biphasic systems[34]. In 1955 Albertsson[35] mixed polyethylene glycol (PEG) with an hydroxylapatite sediment containing firmly-adsorbed chloroplast particles in 1M potassium phosphate buffer. A two-phase system was created and the intense green colour of chloroplasts was present in the top PEG rich phase. That was the first example of using a two-phase system for the partitioning of cell particles.[36]

Nowadays aqueous two-phase systems are commonly used both for single batch extraction[37] as well as a phase systems in countercurrent chromatography[38]. The high water content (70-90%) and very low interfacial tension (0.0001-0.1dyne/cm compared to 1-20dyne/cm for water-organic solvents)[39] makes those systems a comparatively gentle environment for biological materials and thus appropriate for the separation of biomolecules, cellular organelles and whole cells.

a) Partitioning behaviour of amino acids in ATPS

Shang *et al*[40] (2004) made a complex study of the partitioning behaviour of four different amino acids (cysteine, phenylalanine, methionine and lysine) in 15 aqueous two-phase systems made of different PEG molecular weight and potassium phosphate at various pH. Hydrophobicity, electrostatic interaction and pH of a phase system were found to be the main factors affecting the partitioning of amino acids in ATPS. The more hydrophobic PEG-phase was favoured by the non-polar phenylalanine, whose distribution ratio was the highest of the tested amino acids. The important parameter was the charge of the amino acid. Lysine, which at the tested pH was positively charged, preferred the more polar, salt-rich phase. This was explained by the biggest electrostatic interaction being between the lysine cation and the salt anion. The remaining three amino acids, which were negatively charged at the tested pH, preferred the polymer rich phase, because of the repulsion caused by the salt anions. pH positively influenced the partitioning of all amino acids. In most experimental

conditions, especially at high pH, the four amino acids partitioned more into the PEG phase as the molecular weight of PEG increased.

Cohen *et al*[41] (1995) observed a positive correlation between the number of methylene groups in the aliphatic chain of an amino acid and its partitioning in ATPS made of PEG8000 and potassium phosphate. The longer the aliphatic chain of an amino acid, the less polar was the molecule and so the amino acid prefered the less polar, PEG-rich, upper phase.

b) Partitioning behaviour of proteins in ATPS

Partitioning of a protein in ATPS depends on its own properties, such as size, net charge and hydrophobicity, as well as the type of the phase system, such as the polymer and salt type, their concentration, polymer molecular weight and pH.[42]

An aqueous two-phase system, used for partitioning of biomolecules, is usually made of polyethylene glycol (PEG) and dextran or a salt, which usually is a phosphate or a sulphate. In the literature there are other systems based on polyethylene glycol. Other examples of the second polymer include a guar gum derivative[43], starch[44,45], maltodextrin[46] or cellulose[47].

Another type of ATPS consists of systems made of thermoseparating polymers, i.e. polymers which above a certain temperature precipitate out of the solution. This makes the recovery of a sample easier which, staying in aqueous phase is separated from the polymer.

Partitioning of proteins in ATPS made of a thermo separating polymers has been intensively studied by Tjerneld and co-workers[48,49,50]. The publication[50], published in 1995, presents the effect of salts and the surface hydrophobicity of five model proteins, bovine serum albumin (BSA), lysozyme, β-lactoglobulin, myoglobin and cytochrome C, on the partitioning in aqueous two-phase systems. The top phase of two-phase systems consisted of either a relatively polar polyethylene glycol (PEG, 100% EO) or copolymers of ethylene oxide (EO) and propylene oxide (PO) in proportions 50%EO/50% PO and 30%EO/70% PO. The more PO in a polymer, the more hydrophobic was the phase system. Reppal PES 200 (hydroxypropyl starch) was used as the bottom phase polymer. Hydrophobicity and the presence of a hydrophilic salt affected most the partitioning of lysozyme, a protein with the highest surface hydrophobicity. It could be easily driven to the top phase by increasing the proportion of PO and the presence of the relatively hydrophobic anion, ClO_4^-. In the publication[49]

published in 2002 the effect of surface exposed amino acids on partitioning of monomeric proteins was demonstrated. Aromatic residues had the strongest effect on the partition coefficient, showing preference to the less polar upper, EO30PO70, phase. On the other hand the presence of charged amino acids on the protein surface enhanced the partitioning of the protein in the lower dextran-rich phase.

Pereira at al[51] tested an aqueous two-phase system made of Ucon 50-HB-5100 (a mixture of ethylene oxide and propylene oxide) and a salt for the separation of endo-polygalacturonase, which partitioned to the salt phase. Then by increasing the temperature of the polymer phase, the polymer was extracted with a recovery of 95%.

1.3.5.3. Aqueous Ionic Liquids two-phase systems

Ionic liquid biphasic systems consist of room temperature ionic liquids (RTILs) and one or more other solvents such as water, a buffer or an organic solvent.

Room temperature ionic liquid is a collective term for salts with a melting point below room temperature. Although they look like viscous organic solvents, they are molten salts made of ions free to move within the liquid volume. The most common cations are dialkylimidazolium, alkylpyridinium, tetraalkylammonium and tetraalkyl-phosphonium. For the anionic component, most researchers are using tetrafluoroborate, hexafluorophosphate, bis(trifluoromethanesulfonyl) inide dicyanimide[52]. By modification of either the cation or anion or both, the physical and chemical properties of a solvent can be changed, thus it is possible to design a tailor-made solvent[53].

Ionic liquids have been widely researched as possible “green” replacements for organic solvents as they have no vapour pressure and extremely low volatility. For that reason, it may be easier to efficiently reuse solvents based on RTIL compared to those based on organic solvents[53]. Moreover the ability to dissolve many compounds and form two-phase systems with many solvents make RTILs ideal solvents for engineering media for biocatalytic reactors[54].

Ionic liquids can be used as a polar environment for enzymatic reactions, replacing conventional polar organic solvents which usually inactivate enzymes. However not all ionic liquids are suitable for biocatalysis. Enzymes usually stay active in RTILs containing BF_4, PF_6 and NTf_2 anions, but not in those containing Cl, NO_3, CF_3SO_3, trifluoroacetate or acetate anions. The most common ionic liquids used in biocatalysis are imidazolium based ionic liquids such as BMIM·BF_4 (1-butyryl-3-

methyl-imidazolium tetrafluoroborate)[53]. Van Rantwijk *et al*[55] have reviewed the effect of different ionic liquids on enzyme activity and the thermal and operational stability of biocatalysis.

Luts-Wahl *et al*[56] tested the activity and stability of free and immobilised D-amino acid oxidase from *Trigonopsis variabilis* in the presence of five different ionic liquids. The most promising RTILs were BMIM·BF_4 and BMIM·$MMPO_4$. However the DAAO activity depended on the water content and the enzyme was the most active in the highest tested water content (80%).

In the literature are reports that some aqueous two-phase systems based on ionic liquids are able to retain proteins in the IL-rich phase[57,58,59].

Since many RTILs make biphasic liquid systems with numbers of solvents, including water, they can be used as part of a phase system in countercurrent chromatography[60]. The biggest challenge is the high viscosity of pure RTILs which can be reduced by adding some organic solvent, such as acetonitrile or a short chain alcohol[61]. The ionic liquid-based ATPS is better retained in a CCC coil compared to the similar PEG-based ATPS, due to the larger phase density difference for the IL-based ATPS [60].

There are already examples in the literature of applying RTILs as solvent systems in CCC.

An ionic liquid, 1-butyl-3-methylimidazolium hexafluorophosphate (BMIM·BF_6), was successfully employed for the first time as a stationary phase in CCC for retaining different aromatic solutes, including bases, acids and neutral compounds[62]. Acetonitrile was added to the phase system in order to reduce the viscosity of the IL.

Ruiz – Angel *et al*[60] (2006) investigated the ATPS based on an IL, BMIM·Cl and K_2HPO_4 as the phase system in CCC for the separation of four model proteins (cytochrome C, haemoglobin, myoglobin and ovalbumin). However, the separation was found to be not possible, as all four tested proteins were totally partitioned into the BMIM·Cl rich phase.

1.4. Chemistry of chiral compounds

Isomers are different compounds that have the same molecular formula but different structural formula. Isomers that have the same connectivity but differ in the arrangement of their atoms in space are stereoisomers. If a molecule lacks an internal plane of symmetry and has a non-superimposable mirror image like a left and a right hand, it is called a chiral compound (from the Greek χειρ, hand). Two mirror images of a chiral molecule are called enantiomers (from the Greek ἐνάντιος, opposite, and μέρος, part or portion). Stereoisomers that are not mirror images of each other are called diaseteroisomers. When two diastereoisomers differ from each other at only one stereocenter, they are epimers.

The existence of a compound in two different chiral forms is often caused by the presence of a chiral carbon atom which is attached to four different atoms or four different groups of atoms. For instance in an amino acid, the amino and the carboxyl group as well as hydrogen and a substituent are attached to the same carbon atom which makes it chiral (Figure 11). Therefore, most amino acids, including amino butyric acid, exist as two chiral forms. An equimolar mixture of two enantiomers is called a racemic form (either a racemate or a racemic mixture).

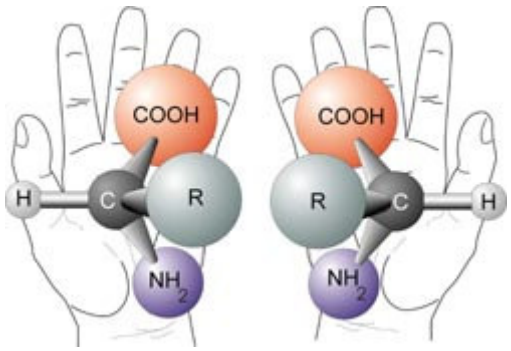


Figure 11 Chiral D- and L-amino acids image [from http://www.nai.arc.nasa.gov/]

Most physical properties of a pair of enantiomers, such as boiling point, melting point, solubility, refraction index, infrared spectra and solubility in common solvents, are identical. The physical property that differentiates a pair of enantiomers is an interaction with plane-polarised light. The plane of plane-polarised light is rotated when the light passes through a solution of an enantiomer. Two enantiomers always

show opposite optical activity; that is, the plane of plane-polarised light passing through an equimolar solution of an enantiomer is rotated at the same angle but in the opposite direction for the two enantiomers. Dextrorotatory enantiomers, also labeled (+), rotate the plane of the polarized light in the clockwise direction, whereas their mirror-images, levorotatory enantiomers, labeled (−) rotate the plane of the polarized light in the counter clockwise direction.

The important system of nomenclature for denoting enantiomers is the *R / S* system, which labels each chiral center *R* or *S* according to a system in which its substituents are each assigned a *priority*, according to the Cahn-Ingold-Prelog priority rules. The chiral centre of a molecule needs to be oriented so that the lowest-priority of the four is pointed away from a viewer. If the priority of the remaining three substituents decreases in clockwise direction, it is labeled *R* (for *Rectus*), if it decreases in counterclockwise direction, it is *S* (for *Sinister*).

In certain areas of biochemistry, such as amino acid and carbohydrate chemistry, the D/L system is used by relating a molecule to either D- or L-glyceraldehyde.

A wide range of biological and physical functions are generated through precise molecular recognition because enzymes, receptors and other natural binding sides interact with different enantiomers in decisively different ways[63]. For that reason molecular chirality plays a fundamental role in many biological processes. For example, the dextrorotatory enantiomer of limonene has the odour of orange, but the levorotatory enantiomer smells of lemons. One enantiomer of a compound called carvone is the essence of caraway, the other, the essence of spearmint. This shows that the two enantiomers of the same compound can stimulate different receptors.

Chirality is especially important in pharmacology, for many drugs used in pharmacology both enantiomers are active. However it often happens that only one form (eutomer) shows a pharmacological effect, whereas the other enantiomer (distomer) might cause several effects such as toxicity, inactivity or an antagonistic effect. Thus, especially if one of the enantiomers shows toxicity, only the desired form can be applied.

For instance ibuprofen contains one chirality centre, and only the S-enantiomer is active as an analgesic and anti-inflammatory agent. The R-enantiomer of ibuprofen is inactive, although slowly converted in the body to the active S form. A medicine based on the S-form alone takes effect more quickly than a racemate.

Thalidomide, a drug which was used in early 1960's to alleviate the symptoms of morning sickness in pregnant women, exists in two enantiomeric forms. One of them had the desired therapeutic effect, but the other was the cause of birth defects in many children.

This shows the significance of using the correct enantiomer, especially in medical applications. In many cases the synthesis of a chiral compound leads to an equimolar mixture of two forms that later need to be resolved.

1.4.1. Enantioseparation (chiral resolution)

Enantioseparation, called also chiral resolution, is a collective term for the techniques used to obtain a pure enantiomer. A sample of an optically active compound that consists of a single enantiomer is said to be enantiomerically pure or to have an enantiomeric excess of 100%. The enantiomeric excess (ee) is defined as:

$$ee = \frac{moles\ of\ one\ enantiomer - moles\ of\ another\ enantiomer}{total\ moles\ of\ both\ enantiomers} \times 100 \quad \textit{Equation } 6$$

Usually, there are three possibilities to obtain pure enantiomers:

- natural source
- asymmetric synthesis
- separation from a racemic mixture

The limitation of the first method is due to the limited numbers of chiral compounds that can be found in natural sources. Asymmetric synthesis is both expensive and time consuming.[64] Therefore, the most popular method of obtaining pure enantiomers is based on the separation of a racemates.

1.4.2. Characterisation of chiral separation techniques

There are several methods available for separation of chiral compounds:

- Crystallization
- Membrane separation
- Kinetic resolution
- Chromatography
    - Simulated moving bed

- Liquid – liquid extraction
- Liquid-liquid chromatography (CCC)
- Gas chromatography
- Capillary electrophoresis
- Liquid chromatography (HPLC)

1.4.2.1. Resolution of the racemate through crystallisation

Two enantiomers can interact with a reagent to form a new complex compound of diastereomers. Diastereomers are stereoisomers that are not enantiomers, therefore having different physiochemical properties, one can be selectively crystallised through traditional techniques. That is called diastereomeric crystallisation.

In 1849 Louis Pasteur, a French chemist and biologist, separated the two enantiomers of tartaric acid by the crystallisation of salts of tartaric acid derived from wine. That led to the discovery of enantiomerism. Salts of tartaric acid form two distinctive kinds of crystals which could be separated using tweezers and a magnifying glass.

Unfortunately, few organic compounds give chiral crystals as do the (+) and (-) tartaric acid salts. Therefore, Pasteur's method is not one that is generally applicable. The selection of an appropriate resolving agent remains a serious challenge. There are, however, a few examples in the literature of using diastereomeric crystallisation. For instance a racemate of mandellic acid was enriched up to 85% by the use of L-phenylalanine as the resolving agent[65].

1.4.2.2. Membrane technology in enantioseparation

Very promising new processes are enantioseparations using the membrane separation methods[66]. A chiral selector membrane binds one of the enantiomers, while allowing the other enantiomer to pass through the membrane. In principle, the membrane separates one enantiomer from the chiral solution to a pure solvent. The technique can be divided into two main classes: the chiral selector molecules are located in retentate phase or inside the of porous, solid membrane layer. The membrane itself can be enantioselective, or an enantioselective carrier is bound to a nonselective membrane.[67] Hadik *et al*[68] (2005) used N-3,5-dinitrobenzoyl-L-alanine-octylester as a chiral selector and a polypropylene hollow fiber membrane for the resolution of DL-lactic acid. Jiao *et al*[69] (2006) presented enantioseparation of

salbutamolsulfate on polyvinylidene fluoride hollow fiber membrane using tartaric acid ramifications D-dibenzoyltartaric acid and di-*p*-toluoyl-D-tartaric acid as combinatorial chiral selectors. Ul-Haq *et al*[70] (2008) used an enantioselective membrane made of poly[acrylonitrile-co-acrylic acid] for enantioseparation of phenylalanine. Gumi *et al*[71] proposed a membrane-based system for enantioseparation of β-blocking drug, SR-propanolol, using N-hexadecyl-L-hydroxyproline as a chiral selector.

1.4.2.3. Kinetic resolution in enantioseparation

Kinetic resolution is applied to two enantiomers which show different reaction rates in a chemical reaction, thereby creating an excess of the less reactive enantiomer.

An important part of enantioseparation is a dynamic kinetic resolution using various enantioselective enzymes which, by converting only one of the two enantiomers, leaves the other unaltered. Then the unaltered form, which is usually a target compound, is isolated using a chromatographic or separation technique. The theoretical maximum yield of the desired form of an enantiomer is 50%. The yield can be increased even up to 100% in the case of applying a racemase[72], an enzyme which catalyses stereochemical inversion around an asymmetric carbon atom.

There are examples of performing an enzymatic kinetic resolution in a membrane bioreactor for enantioseparations. In 2009 Ahmad *et al*[73] proposed a membrane bioreactor for the production of L-homophenylalanine. Ong *et al*[74] in 2008 studied the enantioseparation of (R,S)-ketoprofen using *Candida antarctica* lipase B, which catalysed the esterification of (R)-ketoprofen, leaving the target compound (S)-ketoprofen in unreacted form. At the optimum reaction conditions, the conversion of (R)-ketoprofen was 73%, with an enantiomeric excess of the product (ee) of 87.8%. Unlike the techniques described in section 1.4.2.2, when enantioselective membranes were used, membranes used in the membrane bioreactor provided only an essential barrier to enzymes, keeping them in soluble form, while allowing the transport of substrates and products to and from the homogenous solution.

Koter and Ceynowa[75] in 2003 described the kinetic resolution of chiral alcohols in a bioreactor based on a bifunctional membrane made of asymmetric polyamide film. The membrane exhibited both enantioselective permeabilities and catalytic properties

induced by the immobilised lipase. Applying a bifunctional membrane produced an increase of the enantiomeric excess (ee) of the product from 80%, when a nonselective membrane with an immobilised enzyme was used, to 98.5%.

1.4.2.4. Chromatography in enantioseparation

Two enantiomers can be separated from each other using chromatographic techniques. As described in section 1.2.1, chromatography is a separation technique which is based on the different partitioning of compounds between the stationary and the mobile phase. Since two enantiomers share the same physical properties, they both partition between the two phases to the same extent. Therefore, to make a separation possible, one of the two following methods is applied. A direct method requires a chiral stationary phase (CSP) or a chiral selector if an achiral stationary is used. Indirect methods involve a chiral derivatising agent (CDA) which, upon reacting with the enantiomers, gives diastereomeric derivatives which can be separated on an achiral stationary phase[76].

1.4.2.5. Simulated moving bed in enantioseparation

A common technique used for enantioseparations is simulated moving bed (SMB). The system consists of several columns (usually 6-12) connected in series. Due to the movement of the external streams and internal flow-rates of sections the counter-current flow of the absorbent is simulated.

Schulte and Strube[77], in their review of 2001, quote a number of chiral compounds with enantiomers which were separated using SMB. Among them are DL-threonine, *cis/trans*-phytol, 1-phenylethanol, cyclosporine A, aminoglutethimide.

Swarup *et al*[78] used tris cellulose 3,5 dimethylphenyl carbamate bonded on silica gel, the chiral selector, to resolve oxprenolol.

1.4.2.6. Enantioselective liquid-liquid extraction

Enantioseparations based on centrifugal contactor separators (CCS) have been proposed by Schuur *et al*[79, 80] in 2009. Since a single centrifuge can provide only one mixing and separating step, several stages are required to obtain 99% ee, the generally accepted measure of “pure” enantiomer. For the enantioseparation of phenylglycinol with an azophenolic crown ether as a chiral selector, 4 stages were sufficient, whereas

12 stages were required for the separation of 3,5-dinitrobenzoyl-leucine using a cinchona alkaloid derivative. In both cases racemates dissolved in the aqueous mobile phase entered the extraction cascade made of a number of CCS connected in series. The enantiomers were countercurrently flowed with the organic stream containing the extractant. The organic phase was enriched with the (S)-enantiomer due to a preferential extraction of this enantiomer. Then the organic stream containing (S)-enantiomer was back-extracted to recover the extracted enantiomer and to recycle the organic phase with an extractant.

1.4.2.7. CCC in enantioseparation

The main advantage of countercurrent chromatography over solid-liquid chromatography comes from the lack of a solid support. One consequence of this is the availability of a considerable proportion of the stationary phase volume, up to 80% or even 90%, compared to a maximum of 20% for reversed phase high performance liquid chromatography (HPLC). Since the stationary phase in CCC is also liquid, a separated analyte has access to the whole volume of the stationary phase, unlike in HPLC where it is limited to the interface with the mobile phase. This leads to a higher loading capacity and a lower solvent consumption.[81] Moreover, the chiral selector (CS) can be re-extracted from the liquid stationary phase by common separation techniques such as evaporation of the liquid phase.[82]

However, in spite of the mentioned advantages, this technique also has some limitations, especially if applied to enantioseparations. The major difficulty is to find a chiral selector that is highly selective in a liquid phase as well as a combination of solvents that do not destroy this selectivity yet retain the capacity to elute the chiral isomers of interest.[83] Moreover, the CS must totally partition into one of the two phases, used as the stationary phase. Otherwise, the continual stripping of the CS with the mobile phase would contaminate the eluting enantiomer. Due to these difficulties, examples of enantioseparations using countercurrent chromatography are not numerous[84].

In 1980's pioneering work on applying counter-current chromatography for enantioseparations was performed on low efficiency locular counter-current chromatography (RLCC) and droplet counter-current chromatography (DCCC) [85]. It was often taking a few days to complete a separation of a pair of enantiomers. The technique was applied by Domon and co-workers[86] (1982) for the separation of

enantiomers of (±)-norephedrine using RLCC consisting of 16 columns, divided into 37 loculi each. (R,R)-di-5-nonyl tartarate was used as a chiral selector. The complete separation was achieved after four days. Although the separation technique was very slow, the potential of CCC as a tool for chiral separations was demonstrated. Oya and Snyder[87] (1986), used DCCC for the chiral resolution of carboxylic acid with (-)-R-2-aminobutanol as CS. The resolution was achieved after 56 hours. In another application of DCCC, resolution of DL-isoleucine using N-n-dodecyl-L-proline as a chiral selector took 2.5 days.[88]

The introduction of high speed counter-current chromatography (HSCCC) enabled the separation time of enantiomers to be shortened to 1-2 hours which has made the technique more popular[89]. One of the earliest chiral separations using HSCCC[90] was performed in 1995 to resolve (±)-dinitrobenzoyl amino acids (DNB-phenylglycine, DNB-phenylalanine, DNB-valine, DNB-leucine). N-dodecanoyl-L-proline-3,5-dimethylanilide was used as a chiral selector. In this study, standard CCC and pH-zone refining techniques were compared. By using a standard CCC method, from 10mg to a maximum of 1g of samples were resolved in 2-9 hours, using a solvent system consisting of hexane-ethyl acetate-methanol-10mM hydrochloric acid. To achieve higher loading, a more concentrated CS was used. By using pH-zone-refining, more sample (2g) was efficiently separated in less time (3h), using a phase system based on *tert*-butyl ether-water. It was also demonstrated[89] that increasing the concentration or net amount of CS in the stationary phase improved both the separation factor and peak resolution.

Since then various chiral selectors and operational modes have been proposed for operating CCC. The most used group of CS has been L-proline and its derivatives. As mentioned above, N-dodecanoyl-L-proline-3,5-dimethylanilide was also used to resolve of N-(3,5-dinitrobenzoyl)-tert-butylleucinamide and N-(3,5-dinitrobenzoyl)-tert-butylvalinamide[91] as well as N-(3,5-dinitrobenzoyl)-(±)-leucine[92], whereas L-proline and (4R)-hydroxy-L-proline were used for the chiral separation of N-(3,5-dinitrobenzoyl)-(±)-leucine and (±)-ketoprofen[93].

Cinchona-derived anion-exchange has been adopted in CCC for the separation of enantiomers of N-derivatised amino acids and 2-aryloxypropionic acid[94].

β-cyclodextrin was applied for the separation of 7-des-methyl-ormeloxifene[95], whereas its derivative, carboxymethly-β-cyclodextrin was applied for the chiral

resolution of aminglutethimide[96] and chlorpheniramine[97]. Cellulose tris(3,5-dimethylphenylcarbamate) was used for the separation of pindolol and warfarin[98,99].

The racemate of gemifloxacin was resolved using crown ether, (+)-(18-crown-6)-tetracarboxylic acid[100,101] as the CS.

There are also examples of using proteins as a chiral selector for enantioseparations in counter-current chromatography. Bovine serum albumin was used to resolve racemates of DL-kynuremine[102] and DL-tryptophan[103].

Rubio *et al*[104] applied (S)-naproxen N,N-diethylamide as a chiral selector for the resolution of (±)-N- (3,4 –cis -3 -decyl -1,2,3,4 -tetrahydrophenanthren -4-yl) -3,5 -dinitrobenzamide in multiple dual-mode (MDM) using CCC. The CCC coil was initially filled with the lower phase of the solvent system consisting of n-hexane-ethyl acetate-methanol-water (9:1:9:1, v/v/v/v), containing the CS. This phase was the initial stationary phase. The sample was injected onto the equilibrated column. The mobile phase was the upper phase pumped from head to tail direction. When one of the enantiomers was about to elute, the mobile phase as well as the flow direction were altered, thus the lower phase with the CS become the mobile phase, pumped through the column from tail to head direction. To avoid elution of analytes with the lower phase where the CS was partitioned, the system was then returned to the initial conditions, thus the mobile phase once again become the upper phase pumped from head to tail of the coil. Both enantiomers eluted separated from each other and free of the chiral selector. Applying MDM allowed the resolution between the two peaks to be improved from 0.85, when a classic elution mode was applied, to 1.51.

1.4.3. Production of 2-L-aminobutyric acid (L-ABA)

L-(+)-2-aminobutyric acid (L-ABA) and D-(-)-2-aminobutyric (D-ABA) are the racemic forms of 2-aminobutyric acid (DL-ABA). L-ABA (Figure 12) like many other unnatural amino acids[105] is obtained as a result of synthesis.

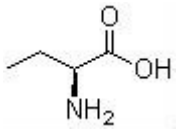


Figure 12. L-(+)-2-aminobutyric acid chemical structure

In 1988, Tokarski *et al*[106] obtained 2-L-aminobutyric acid in a reaction catalysed by a transaminating enzyme with 2-ketobutyrate and alanine.

2-L-aminobutyric acid has also been synthesised from 2-ketobutyric acid and benzylamine in the enzymatic reaction catalysed by ω-transaminase. The reaction was performed in an aqueous-organic biphasic system as benzaldehyde, the co-product of the reaction, inhibited the enzyme. Over 99% of enantiomeric excess of 2-L-aminobutyric was achieved.[107]

Ingenza Ltd (Wallace Building, Roslin BioCentre, Midlothian, EH25 9PP, UK) used the reduction of ketobutyric acid (KBA) for the production of 2-L-aminobutyric acid. This process is nonselective, leading to a racemate of 2- aminobutyric acid. To separate the two forms, the unwanted D-form was oxidised by the enantioselective enzyme D-amino acid oxidase (section 1.5.3) to KBA, whereas L-ABA stayed unaltered. Then L-ABA was separated from KBA in a two-phase batch extraction. The enantiomeric excess (ee) of 2-L-aminobutyric acid obtained was over 99.5%.

1.5. Biocatalysis

Biocatalysis is the use of natural catalysts, such as protein enzymes or RNA enzyme also called ribozyme, to perform a chemical transformation on organic compounds. In this project CCC was used as a bioreactor with the biocatalyst immobilised in the liquid stationary phase. The biocatalyst used was a protein enzyme, D-amino acid oxidase.

1.5.1. Structure of enzymes

Enzymes[108] are biological catalysts which increase the rate of chemical reactions without themselves suffering any overall change. The reactants of enzyme-catalysed reactions are termed substrates and each enzyme is quite specific in character, acting on a particular substrate or type of substrates.

Although nearly all enzymes are proteins, many of them contain a non-protein component called a *cofactor*. The protein part of the enzyme is termed *apoenzyme*, whereas the whole structure is called a *holoenzyme*. A further subclassification of cofactors has been introduced according to how tightly they are bound to an enzyme.

Coenzymes are loosely-bound cofactors whereas a prosthetic group is bound so tightly that it is difficult to remove without damaging the enzyme.

1.5.2. Enzyme protein structure

Since enzymes are proteins, four levels in their structure can be determined. The primary structure of the protein is the sequence of amino acids, which determines its unique properties. Secondary structure refers to regularly repeating local structures stabilized by hydrogen bonds. The tertiary structure is the overall shape of a single protein, often stabilised by covalent bonds between two thiol groups of cysteine amino acids. The term quaternary structure refers to the arrangement of the subunits in oligomeric enzymes, as most large proteins consist of a number of subunits held together by non-covalent forces.

α-Amino acids are classified on the properties of the side chain into four groups: polar, nonpolar, basic and acidic. Prevalence of one type of -amino acid in a protein molecule determines the protein's polarity and net charge at a certain pH.

1.5.3. D-amino acid oxidase (DAAO)

D-amino acid oxidase (DAAO, EC 1.4.3.3.) is a peroxisomal enzyme belonging to the class of flavin dehydrogenase as it contains non-covalently bound flavin adenine dinucleotide (FAD) as the prosthetic group. The enzyme is expressed in a wide range of species from yeasts to human. It is not present in bacteria or in plants. DAAO catalyses an O_2-dependent transformation of D-amino acids to the corresponding α-imino acids, which spontaneously hydrolyze to α-keto acids, ammonium ions and hydrogen peroxide (Figure 13).

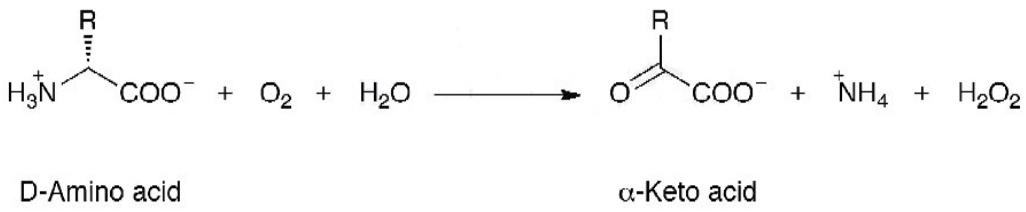


Figure 13 A reaction catalysed by D-amino acid oxidase.

DAAO from pig kidney and from yeast possess a broad substrate specificity. However it is not active against acidic amino acids such as D-glutamate and D-aspartate [109].

The pH optimum of DAAO isolated from the yeast *Trigonopsis variabilis* is between 7 and 9. The enzyme shows the highest activity in the temperature range between 50 and 55°C. However, although the enzyme is stable at temperatures up to 40°C it loses activity when incubated above that temperature and is completely inactivated at 65°C within 30 min[110]. According to Schrader and Andersen[111] (1996) the apparent V_{max} and K_m values for different substrates vary from 3.7 to 185 U/mg and 0.2 to 17.3 mM, respectively.

D-amino acid oxidase is one of the few flavoproteins that have been exploited in enzyme technology. Currently the most important use of DAAO is the conversion of cephalosporin C into 7-(5oxoadipoamido) cephalosporanic acid that is further transformed chemo-enzymatically into 7-ACA (7-aminocephalosporic acid), the precursor of numerous cephem antibiotics[112].

Sacchi *et al*[113] (1998) proposed a biosensor based on DAAO from *Rhodotorula gracilis* for quantifying the presence of bacteria in food. Since D-amino acids are formed mainly by bacteria, the current produced by oxidising D-amino acids is proportional to the bacterial contamination in food.

1.5.4. Monoamine oxidase (MAO)

Monoamine oxidase (MAO) is a flavoprotein that catalyses the oxidative deamination of primary amines into imines, which are then hydrolysed to give aldehydes[114]. Since the enzyme was originally discovered in the liver, it has been found bound to the outer membrane of mitochondria in most cell types in the body.

1.5.5. Bioconversion in two-phase systems

An aqueous two-phase system seems to be an ideal phase system for the CCC-bioreactor because it creates a gentle environment which is known to maintain enzymatic activity. In the literature, there are a number of examples using ATPS as a reaction media. The advantages of performing a bioconversion on such media result from the possibility of enzyme recovery and product extraction (extractive

bioconversion), which plays a key role in case of a bioconversion inhibited by a product of the reaction.

There are a number of publications demonstrating the conversion of benzylpenicillin (penicillin G) to 6-aminopenicillanic acid (6-APA) in various ATPS, when the enzyme, penicillin acylase, was used in both free form and in whole cells of *E. coli* in which form the enzyme turned out to be more stable, allowing ten times repeated batch conversion. To perform the reaction, Liao *et al*[115] (2009) used the phase system made of PEG6000 and potassium phosphate. Xue-jun[116] and co-workers (2004) performed the same reaction in five repeated batches using PEG 20,000 and Dextran T 70 as the phase system. In both experiments, the enzyme was partitioned in the bottom phase, whereas the product was extracted to the top phase. Anderson *et al*[117] (1984) performed the reaction with free penicillin acylase, using ATPS made of 8.9% (w/w) PEG2000/7.6% (w/w) potassium phosphate. The enzyme partitioned into the bottom phase, allowing repeated batch conversion. Applying the enzyme in discrete countercurrent mode allowed Wang *et al*[118] (2007) to achive an increase of the conversion ratio of the reaction from 50 to 65%. Dong-Zhi *et al*[119] (2002) used the enzyme immobilised in 20% (w/w) PEG400/ 15%(w/w) magnesium sulphate for the synthesis of cephalexin from 7-aminodeacetoxicephalosporanic acid (7-ADCA) and phenylglycine methyl ester (PGME).

There are a few more examples of performing an extractive bioconversion on ATPS made of PEG and dextran. Ilic *et al*[120] (2005) performed the bioconversion of apigenin-7-O-β-glucoside into apigenin in polyethylene glycol 6000 / dextran 20,000 by β-glucosidase. PEG40,000/Dextran 40 aqueous two-phase system was used for enzymatic hydrolysis of cellulose[121].

Jyh-Ping and Cheng-Hsin [122] tested various aqueous two-phase systems for lactose hydrolysis with bacterial, yeast and fungal β-galactosidase. The distribution ratio of lactose in ATPS made of 30% (w/w) PEG4000 and 7.5% (w/w) sodium citrate was found to be 0.0042 which allowed for an extractive hydrolysis of lactose. After 84h of hydrolysis in the phase system, the enzyme had lost only 10% of its activity.

Mian and co-workers[123] (2002) tested four varied two-phase systems, PEO-PPO/$(NH_4)_2SO_4$, PEO-PPO/$MgSO_4$, PEG/$(NH_4)_2SO_4$ and PEG/$MgSO_4$ for the bioconversion of starch with α-amylase and amyloglucosidase. The higher production of maltose and glucose was when PEO-PPO/$MgSO_4$ was used.

The extractive bioconversion with the bacterium, *Arthrobacter simplex* was studied by Kaul and Mattiasson[124] (1986) for the transformation of hydrocortisone to prednisolone in aqueous two-phase systems. In order to reduce the cost of dextran, a cheaper starch-based polymer (Reppal-PES) was successfully used.

1.6. Simultaneous conversion and separation

The concept of reactions in chromatographic columns comes from early 1960s[125], when it was applied to reversible chemical reactions, which could be pushed to completion despite an unfavourable equilibrium constant, by constantly removing the products of a reaction.

Chromatographic reactors[126] are made to perform a simultaneous conversion of a compound and removal and separation of formed products during a reaction. This has a great advantage in the case of reversible reactions, where the conversion can be pushed to completion by removal of product, as well as the removal of putative inhibitors. The last case refers to those catalysts which are inhibited or inactivated by a product of a reaction.

Figure 14 shows a schematic batch chromatographic reactor. In a reaction, which takes place in the column, substrate A, injected into a column, is converted onto the products B and C. Because of their different retention times, products B and C are separated from each other as well as from any unconverted compound A.

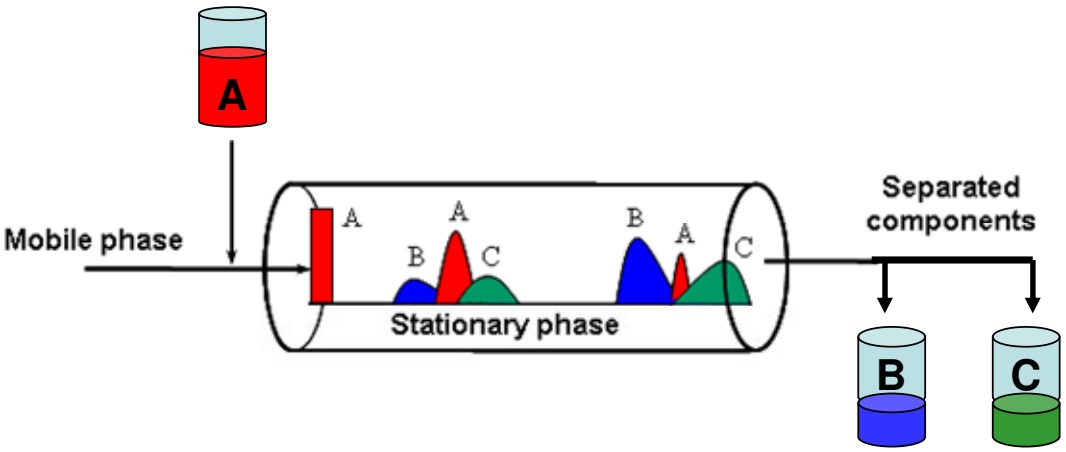


Figure 14 Schematic presentation of the batch chromatographic reactor. A is injected onto a column with the mobile phase. As it travels through the column it is converted into B and C which are separated by the chromatographic action of the column.

1.6.1. Chromatographic bioreactor

Chromatographic bioreactors are chromatographic reactors where an enzyme or an enzyme system, present either in pure form or in a cell preparation, catalyses the reaction. Few chromatographic bioreactor systems have been reported in the literature and the majority of those were performed with an enzyme immobilised on a solid matrix of the stationary phase.

Akintoye *et al*[127] (1991) performed inversion of sucrose to glucose and fructose in the presence of the enzyme invertase in a semi-continuous counter-current chromatographic reactor-separator (SCCR-S) consisting of twelve columns packed with calcium-charged, crossed-linked, polystyrene resin. The enzyme was not immobilised on the column, therefore the reaction occurred in the liquid mobile phase. Purities of fructose and glucose were over 90% and complete conversion was achieved, even at 55% sucrose feed concentration. Subsequently, the process was conducted at a preparative scale with throughput in excess to 16kg of dry sugar per cubic metre of resin per hour, achieving 100% sucrose conversion, and product purities up to 95% w/v. SCCR-S allowed increasing the formation of high molecular weight dextran, at 80% in comparison to conventional process. Dextran can be synthesised as a result of an enzymatic conversion of sucrose. Fructose, which is a side-product of the reaction, inhibits the enzyme dextransucrase. As the bioconversion was performed in SCCR-S, fructose was continuously removed from the reaction media. That resulted in the yield of over 77% of synthesised dextran with a molecular mass of more than 150,000Da. [128]

SCCR-S has also been used for the saccharification of modified starch to maltose and dextrin[129] and hydrolysis of lactose[130].

All these separations were catalysed by a non-immobilised enzyme which was continuously added to the mobile phase.

An HPLC column has also been used as a chromatographic bioreactor for enantioseparations. Calleri *et al*[131] in 2004 presented an enzyme reactor based on β-glucuronidase for *on-line* determination of the urinary molar ratio of dextromethorphan (DOMe) and dextrorphan (DOH). The enzyme was immobilised in an HPLC analytical column, to perform both bioconversion and separation as well as quantification of DOMe and DOH.

Various esterolytic reactions were successfully performed by *Candida rugosa* lipase immobilised on the silica stationary phase in HPLC with a simultaneous separation of products form the unconverted enantiomers[132]. Zeying *et al*[133] (2011) used HPLC to perform asymmetrical enantioseparation of chiral esters hydrolised by *Candida antarctica* lipase B immobilised on silica gel.

1.6.2. CCC-reactor

CCC can potentially perform both a conversion and a separation at the same time if a catalyst is immobilised by partitioning to the liquid stationary phase, with the substrate passing through the column dissolved in the mobile phase. The reaction occurs in the zones of mixing, together with separation of product from substrate, by the chromatographic action of the CCC.

In a series of experiments conducted first on CPC then on CCC by Bellefon *et al*[134] (1998), benzaldehyde was reduced to benzylic alcohol by a water-soluble ruthenian complex immobilised in the aqueous stationary phase. The experiment, performed on CPC, used as a liquid-liquid catalytical reactor, was operated in two ways, in continuous mode and in a plug-flow mode. In continuous reaction mode, where the substrate was constantly pumped on dissolved in the mobile phase, the amount of benzylic alcohol produced in the coil depended inversely on the mobile phase flow rate. In a plug injection mode, the substrate travelled through the column, where it was converted into benzylic alcohol as a result of a contact with the catalyst. Since the substrate and the product of the reaction had different partition ratios between the mobile and the stationary phase, the separation between unconverted substrate and the product created in the coil occurred. Although, over 85% of injected benzaldehyde was converted, the process was found to be limited by the chemistry of the reaction[135]. Moreover, the disadvantage of the process was a large quantity of the catalyst was required for the reaction. Therefore, for further studying the CCC bioreactor a faster reaction was tested[136].

CPC has been used by the same research group[137] as a liquid-liquid chemical reactor for the isomerisation of hexen-3-ol into ethylpropylketone with a water-soluble rhodium catalyst. The plug of substrate was injected with the organic mobile phase into the CPC column. The research revealed inefficient mass transfer between the liquid-liquid phases. Despite this, the CPC-bioreactor seemed to have some advantages over other techniques. Due to a weak axial dispersion in the coil an ideal

plug flow could be assumed. Moreover, due to the chromatographic properties of the CPC, product and substrate can be separated along the reacting volume. Finally, working with pulse injections and sampling, the process can be easily automated.

1.6.3. CCC-bioreactor

Although, in the literature there are few examples of using countercurrent chromatography as a simultaneous bioreactor and a separator, there is no evidence of a successfully created CCC-bioreactor which would provide both a total conversion of a substrate and a complete separation of reaction products.

Bousquet *et al*[138] (1995) employed CCC as a bioreactor, using a biphasic system, 1,1,1 trichloro ethane–aqueous phosphate buffer with a bacterial esterase present in the aqueous stationary phase, for an enantioselective hydrolysis of 2-cyano cyclopropyl-1,1 dicarboxylic acid dimethylester. 500mg of the substrate was injected onto the column filled with the enzyme solution of concentration 7g/L. Hydrolysis of one isomer made it go to the aqueous stationary phase, whereas the unreacted isomer remained in the mobile, organic phase. After 8 hours 80% of enantiomeric purity was achieved. In the publication, there were no details of the CCC centrifuge provided, such as the coil volume or type of machine.

Yamada *et al*[139] (1995) used CPC as a bioreactor with the enzyme β-galactosidase immobilised on the coil by encapsulating it in reverse micelles. Substrates of the bioconversion, o-nitrophenol β-D-galactopyranoside and 4-methylumbelliferyl β-D-galactopyranoside, were continuously pumped onto the column with the mobile aqueous phase. The recovery of galactose, the product of the enzymatic reaction, was more than 85% over a period of 5 hours. In this research, no chromatographic action of CPC was used as the centrifuge was only employed to provide an effective mixing between an aqueous and an organic phase, which resulted in a very good mass transfer between the two phases.

Armstrong *et al*[140] (1990) used CPC as a bioreactor and separator with the enzyme α-chymotrypsin to perform the enantioseparation of DL-tryptophan methyl ester. A plug of the substrate was injected with the organic mobile phase into the CPC column. L-tryptophan methyl ester was hydrolised by α-chymotrypsin immobilised in the aqueous stationary phase. Since the column acted as both a reaction bed and a separating media, formed L-tryptophan was separated from unreacted D-tryptophan methyl ester. An unexpected limitation of the process arose from the instability of the

enzyme in the aqueous - organic phase system. Although the enzyme was dissolved in the aqueous stationary phase, constant contact with the organic mobile phase caused its deactivation by denaturation. Therefore, no total conversion of the substrate was achieved. To overcome the enzyme instability in the aqueous – organic phase system and utilise the catalytic activity of α-chymotrypsin, an external incubation step, preceding the separation, was added. In the external enzyme reactor, the initial racemate of tryptophan methanol ester was hydrolyzed for 5 hours by the enzyme. The reaction mixture was then injected onto the CPC, which in this case acted only as a separator. This not only avoided the enzyme deactivation but also reduced its consumption. A considerably smaller amount of enzyme was required when the reaction was performed in an external chamber than in the CPC unit, where it was dissolved in a larger volume of the stationary phase. Moreover, when the enzyme was present in the stationary phase, the first part of the CPC acted as a bioreactor after which the separation of L-tryptophan created from the unreacted D-form occurred on the remaining plates. Therefore, the separation process was more efficient with a preincubation step, as all of the plates of the CPC were used for the separation. Although there was an excellent separation between L-tryptophan and D-tryptophan methyl ester, there was a small overlap area between the two peaks showing incomplete separation. The authors proposed an automated system for the separation of amino acids based on a reaction column with immobilised α-chymotrypsin followed by the stream of the mobile phase led through a detector to a CPC column to separate compounds.

Hollander and co-workers[141,142] (1998, 1999) investigated a bioreactor based on CPC for the enantioseparation of amino acids. The model reaction was deracemisation of N-acetyl-DL-methionine by acylase I. The performed reaction was reversible, therefore simultaneous separation of the two hydrolysis products, acetic acid and L-amino acid, improved the yield of the conversion by shifting the equilibrium of the reaction. The substrate, a racemic mixture of N-acetyl-methionine, was injected onto the CPC column with the mobile phase. As a result of the selective catalytic activity of acylase I present in the upper, stationary phase of the ATPS (16/16% (w/w) PEG600 and potassium phosphate, pH 7.39), only the L-enantiomer of racemic acylated amino acid was hydrolysed to the L-amino acid and acetic acid. Then, in a chromatographic action of CPC, L-methionine produced in the column and acetic acid and unreacted N-acetyl-D- methionine, were separated, eluting individually. Although

there was an overlap between peaks, N-acetyl-D-methionine eluted mainly as a pure compound. However, the authors reported a small amount of unconverted substrate eluted from the column which would indicate incomplete deracemisation of the injected amino acid. Therefore, not only there was no total bioconversion achieved but also the separation was incomplete.

Hollander and co-workers used CPC as a bioreactor which is not as directly scalable as CCC. Moreover, a CPC unit requires the use of two rotary seals which presents the risk of leakage or cross contamination.

1.7. CCC-bioreactor for isolation of L-2-aminobutyric acid

The aim of this current PhD project was the creation of a simultaneous CCC-bioreactor and separator (Figure 15) for the isolation of a high value, enantiomerically pure form of amino butyric acid (ABA) from the relatively cheap racemate. The enzyme was immobilised by partitioning into the liquid stationary phase and the substrate dissolved in the mobile phase passed through the column. The reaction (Figure 16) occurred in the zones of mixing, together with separation of product from substrate by the chromatographic action of the CCC. Since the KBA produced by enzymatic action and the unreacted L-ABA, which was the target compound, had different partition ratios between the two phases, they eluted at different times.

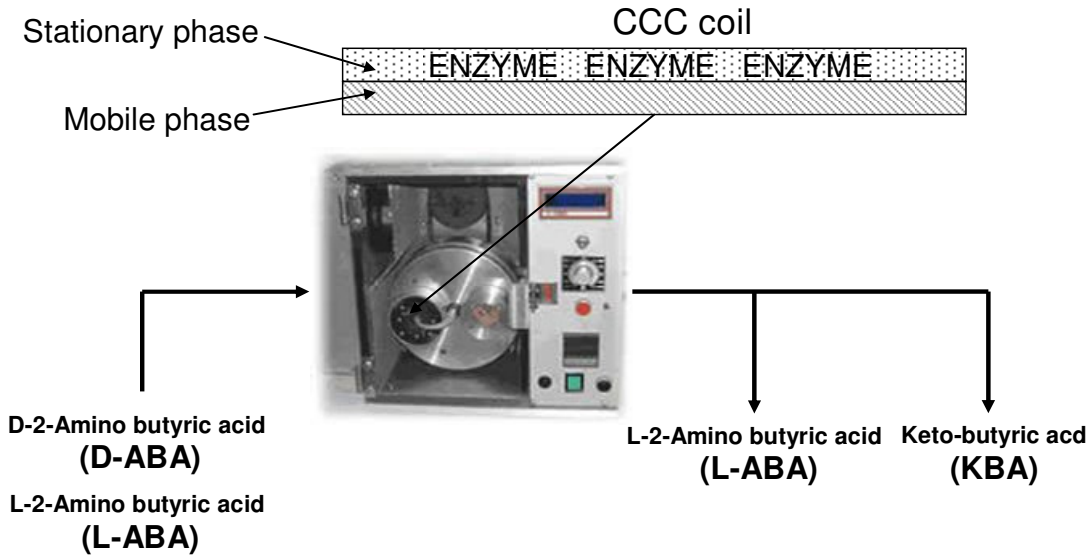


Figure 15 CCC-bioreactor with D-amino acid oxidase in the stationary phase. Only D-form of the aminobutyric acid racemate is oxidised to its keto analog (KBA). Since the L-ABA and KBA have different partition ratios between the two phases in CCC, they elute at different times.

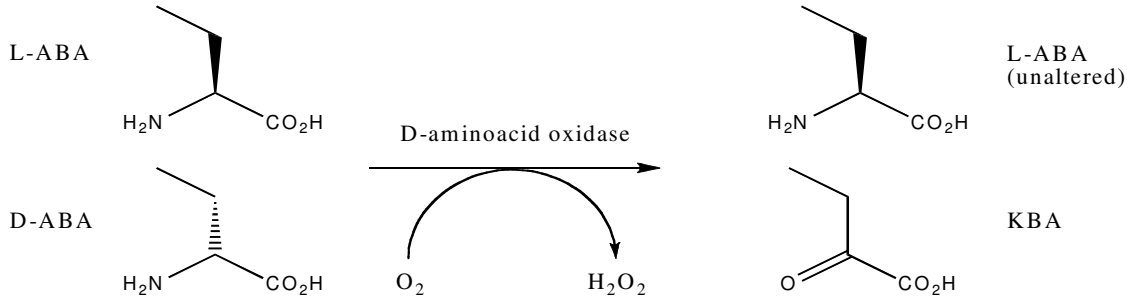


Figure 16. Catalytic action of D-amino acid oxidase (DAAO). D-amino butyric acid (D-ABA) is oxidised to ketobutyric acid (KBA), whereas the other enantiomer L-amino butyric acid (L-ABA) stays unaltered.

2. EXPERIMENTAL SET UP AND DEVELOPMENT OF THE ANALYTICAL PROCEDURES

2.1. Introduction

In this chapter general experimental methods related to this thesis are described.

2.2. Countercurrent chromatography (CCC) experimental set up

Typically, the experimental set up consisted of a CCC Milli centrifuge, an analytical pump, a sample port and a fraction collector. For same experiments a UV-detector was also used. All instruments were connected using 0.8mm bore PTFE tubing.

2.2.1. CCC centrifuge

The J-type CCC centrifuge, called Milli-CCC®, was a prototype machine designed and made by the Brunel Institute for Bioengineering, Brunel University (Uxbridge, UK). The rotor (R) and the planet (r) radii were 50mm and 28-39.5mm respectively, which gives the β-value 0.56-0.75. The centrifuge allowed the column to be rotated at a rotational speed within the range of 500-1800rpm in two modes: clockwise (run) and counter clockwise (empty). In all performed experiments, apart from when the Multiple Dual-Mode (5.3.3.) was applied, the CCC was operated in the run mode during separation and in the empty mode when the coil was being filled with a stationary phase. Filling the coil in the empty mode helps in removing potentially trapped air bubbles.

The CCC column, made at the Brunel Institute for Bioengineering, was made of a PTFE tube, wound around a bobbin in the counter clockwise direction. Table 3 shows the position of the head and the tail of the coil when the coil is rotated clockwise or counter clockwise.

	Periphery	Centre
Spun clockwise	Head	Tail
Spun counter clockwise	Tail	Head

Table 3 Positions of Tail and Head at centre or periphery for a counter clockwise wound coil spun clockwise or counter clockwise.

The bore of a tube is relatively large (2.7mm compared to the standard Milli CCC coil bore 0.8mm) to help retain the ATPS. The first column (Column#1) burst during this research project and was replaced by another called Column#2, also made in BIB (Table 4).

Column name	Coil bore (mm)	Coil length (m)	Coil volume (ml)	Rotor radius (mm)	β-value
Column#1	2.7	7.9	45.1	50	0.56-0.75
Column#2	2.7	7.9	45.0	50	0.56-0.75

Table 4 Parameters of the CCC coils used for the bioreactor studies.

The volume of a column was determined by subtracting the weight of an empty coil from the weight of the coil, entirely filled with water. To determine the weight of an empty column, the coil was first flushed with acetone to remove any remaining water, then, the acetone was blown out and dried by pumping through with compressed air. Three measurements were performed and the average taken.

2.2.2. Liquid - handling pump

The pump used was an analytical HPLC pump K-501, KNAUER (Berlin, Germany), designed to be operated at a flow rate 0.1-10ml. The pump was used to deliver the stationary phase, mobile phase and a cleaning solution to the system.
The pump was primed with deionised water before each use, as well as before changing a phase from upper to the lower phase and vice versa.

2.2.3. Sample loop port

The sample loop port, which was used to inject a fixed amount of a sample onto the column, was designed and made especially for these experiments. It was based on a manually operated 6-way selection valve. The sample loop used in most experiments was a 0.8mm bore PTFE tube of a total volume 1.72ml, which was 3.8% of the 45.1ml coil.

2.2.4. Fraction collector

To collect fractions from CCC a fraction collector was used, [KNAUER (Berlin, Germany), model Foxy Jr.]. Fractions were collected in 2ml tubes (Eppendorf).

2.2.5. UV-detector and the signal recorder

The UV-detector used for some experiments was a KNAUER model K-2501. It was connected via an interface box (KNAUER model IF2) with a computer. The software used was the KNAUER "Eurochrom". The mobile phase passed through the UV-detector and the signal was recorded as a chromatogram. The chromatogram recording was started when the switch was manually pressed after a sample injection.

2.3. Centrifugal Partition Chromatograph (CPC) experimental set up

The CPC unit was purchased from Armen Instrument (Vannes, France). It is equipped with two 500ml rotating columns that can be operated either singly (500ml) or combined (1000ml). Each coil consists of 1008 chambers (0.424ml) which gives a the total chromatographically active volume of 429ml. The volume of interconnecting passages between the chambers is 71ml.

The unit is equipped with two pumps. One of them, the binary built-in, twin headed pump, is used for pumping the upper and the lower phase. The other pump allows a sample injection on the column. There is also an option to use a sample loop instead of a pump, and that option was used in this study. The sample loop was a 23.5ml PTFE tube.

A system of valves allows switching between descending and ascending operation mode.

2.4. Materials

2.4.1. Aqueous two-phase system (ATPS)

All aqueous two-phase systems (ATPS), which were used for the bioreactor, were composed of polyethylene glycol (PEG) and a salt. The concentration of PEG and salt in ATPS was expressed as weight by weight % i.e. 14/14% PEG/salt means that in 100g of ATPS there is 14g PEG, 14g salt and 72g water. Thus to make ATPS, both PEG and salt were weighed out on a balance (accuracy 0.01g). PEG1000 exists in a solid form, therefore before use, it was melted at +60°. Both PEG3350 and PEG8000 are powders, whereas PEG400 is a liquid. All salts used were powders, thus they were weighed out in a separate beaker and transferred to the previously weighed PEG. Finally a desired amount of deionised water (purity 1.0MΩ cm^{-1}), warmed up to 50-60°C was added. Then all was mixed until the salt and PEG were entirely dissolved. A freshly made ATPS was left, usually overnight, in a separating funnel to bring it to room temperature. It was then shaken again and after phase separation the lower phase was run off. Each phase was stored in glass bottles in a dark place at room temperature for a period no longer than a month.

Chemicals used for ATPS	CAS	Supplier
Polyethylene glycol mol wt. 400	25322-68-3	Sigma-Aldrich
Polyethylene glycol mol wt. 1000	25322-68-3	Sigma-Aldrich
Polyethylene glycol mol wt. 3350	25322-68-3	Sigma-Aldrich
Polyethylene glycol mol wt. 8000	25322-68-3	Sigma-Aldrich
Ammonium sulphate	7783-20-2	Fisher
Potassium sulphate	7778-80-5	Sigma-Aldrich
Sodium sulphate	7757-82-6	Sigma-Aldrich
di-Ammonium hydrogen orthophosphate	7783-28-0	Fisher
Ammonium di-hydrogen orthophosphate	7722-76-1	Fisher
di-Potassium monohydrogen phosphate	7758-11-4	Fisher
Potassium dihydrogen phosphate	7778-77-0	Fisher
di-Sodium monohydrogen phosphate	7558-79-4	Fisher
Sodium dihydrogen phosphate	13472-35-0	Fisher
Potassium citrate	6100-05-6	Sigma-Aldrich
Sodium citrate	6132-04-3	Sigma-Aldrich
Citric acid	5949-29-1	Sigma-Aldrich

Table 5 List chemicals and their suppliers used to make ATPS.

2.4.2. Enzymes tested for the CCC-bioreactor

2.4.2.1. D-amino acid oxidase (DAAO)

D-amino acid oxidase (DAAO) was provided by Ingenza Ltd (Wallace Building, Roslin BioCentre, Midlothian, EH25 9PP, UK) in the free form and in whole cells of *Escherichia coli*.

DAAO was originally isolated from *Trigonopsis variabilis* and expressed in thermal induced *Escherichia coli* in a liquid fermentation. The enzyme's expression was preceded by screening procedures aimed to improve its activity and stability. After the fermentation, cells were centrifuged and the pellet, consisting mainly of *E. coli* cells, was either disrupted to obtain the free enzyme or used as the enzyme in whole cells. To obtain the free form of the enzyme, the pellet was dissolved in water in proportion 1:4 w/w and disrupted by applying a high pressure disruptor (16kpsi), and then centrifuged (30min, 10,000 g). The enzyme protein was in the supernatant, which was directly labelled as "free enzyme".

All those procedures were performed by Ingenza Ltd. The enzyme in the free form was sent to BIB frozen and stored at -20°C. For each experiment some of the enzyme supernatant was defrosted and stored in a fridge at +4°C, no longer than a month. The enzyme in the whole cells was received frozen in dry ice and stored at -80°C.

2.4.2.2. Mono amino acid oxidase (MAO)

Monoamine oxidase (MAO) was provided by Ingenza in the whole cells of *E. coli.* The enzyme was originated from *Aspergillus niger* and expressed in *E. coli.* For experiments the enzyme was used both in whole cells and the free form which was isolated from *E. coli* cells.

Isolation of MAO proteins from *E. coli*

In order to isolate MAO protein from *E. coli,* cell were lysed in the presence of BugBuster®, benzonase and lysozyme.

13.6mg of *E. coli* cells was suspended in 150µL of BugBuster® (Novagen, cat. No 70584), 1µl of benzonase (Sigma-Aldrich, CAS 9025-65-4) 20U/µl and 10µl of lysozyme (Sigma-Aldrich, CAS 12650-88-3) (15mg/ml) and incubated at 37°C for 20min. The clear lysate was centrifuged for 10 min at 13k rpm (approximately 7560g). The supernatant, which contained most of the enzyme protein, was gently removed from the pellet.

2.4.3. Substrates for enzymatic reactions

2.4.3.1. Substrate and product for DAAO

For the enzymatic reactions, DL-aminobutyric acid (CAS 2835-81-6) and D-aminobutyric acid (CAS 2623-91-3) were used. L-aminobutyric acid (CAS 1492-24-6) and ketobutyric acid (CAS 600-18-0) were used as standards. All those chemicals were purchased from Sigma-Aldrich.

2.4.3.2. Substrates and products for MAO

2-phenylpyrrolidine was purchased from Apollo Scientific Ltd. Amylamine (CAS 110-58-7) and α-methylbenzylamine (CAS 618-36-0) were purchased from Aldrich.

2.4.4. Reagents for ABA and KBA derivatisation

ortho-Phthalaldehyde (CAS 463-79-8) and phenylhydrazine (CAS 100-63-0) were purchased from Sigma-Aldrich, and N-isobutyryl-L-cysteine (CAS 124529-02-8) from Merck. Boric acid (10043-35-3) and potassium hydroxide (1310-58-3) were purchased from Fisher Scientific.

2.5. Development of the Analytical Methods

In this section methods developed to optimise and monitor the CCC-bioreactor are presented. These methods are: ABA and KBA HPLC analysis (2.5.1), enzyme activity analysis (2.5.2), enzyme stability analysis (2.5.3) and enzyme concentration analysis (2.5.4).

2.5.1. Analytical methods for ABA and KBA

Analytical methods for both ABA and KBA analysis were developed for two reasons: to determine substrate consumption and product formation in the CCC-bioreactor as well as to monitor the elution time of unreacted L-ABA and produced KBA. Figure 17 shows analytical methods developed to analyse ABA and KBA. Two methods were developed for ABA analysis, a derivatisation with *ortho*-phthaldehyde and a direct analysis on a hydrophilic interaction column (HILIC). For KBA three methods were developed, a derivatisation with phenyl hydrazine and two direct

analysis methods on anion exchange and HILIC columns. The last method can be used to analyse both ABA and KBA.

Each method had its own particular advantages and disadvantages, which are listed in Table 6.

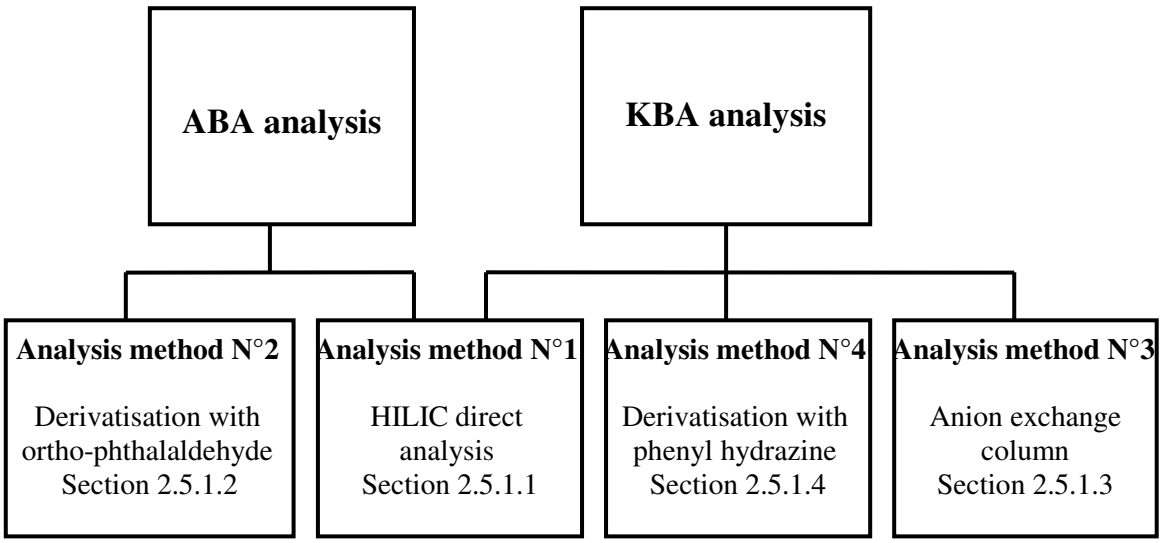


Figure 17. Analytical methods of ABA and KBA

Analysed compound	Method name	Method No	Section number	Advantage	Disadvantage
ABA	Derivatisation with ortho-phthalaldehyde	2	2.5.1.2	Very high sensitivity Enables analysis of both chiral forms	Time consuming derivatisation
	HILIC direct analysis	1	2.5.1.1	No derivatisation needed Both ABA and KBA can be analysed at the same time	Does not distinguish enantiomers of ABA Low sensitivity Not suitable to ATPS
KBA	Derivatisation with phenyl hydrazine	4	2.5.1.4	Very high sensitivity	Time consuming derivatisation
	Anion exchange column	3	2.5.1.3	No derivatisation needed	Low sensitivity Short lifetime of a column

Table 6 Advantages and disadvantages of ABA and KBA analytical methods

All analyses on KBA and ABA were performed on HPLC: Waters 2695 Alliance with Waters 2996 photodiode array detector operated using Waters Empower™ software (Waters Elstree, Hertfordshire, UK).

2.5.1.1. Analysis Method N°1: Direct ABA and KBA analysis on a HILIC column

The HILIC column (page 14) allows the analysis of very polar compounds, thus both the substrate (ABA) and the product (KBA) of the enzymatic conversion could be quantified at the same time. Moreover it is a simple analysis as no derivatisation of a sample is required.

The SeQuant ZIC®-HILIC column, which is a hydrophilic interaction chromatography column, was purchased from Hichrom Ltd. The column length was 150mm and the internal diameter 4.6mm. Particle size was 5µm and pore size 200Å.

To separate ABA and KBA a method named "Ingenza SeQuant ACN gradient" was developed (Table 7).

	Time [min]	Flow [ml/min]	%A	%B	%C	%D	Gradient
1		1.00	0.0	100.0	0.0	0.0	
2	0.10	1.00	0.0	100.0	0.0	0.0	Linear
3	5.00	1.00	100.0	0.0	0.0	0.0	Linear
4	8.00	1.00	100.0	0.0	0.0	0.0	Linear
5	9.00	1.00	0.0	100.0	0.0	0.0	Linear

Table 7 HPLC conditions to quantify ABA and KBA on the HILIC column. Line A: water, Line B: acetonitrile, Method named Ingenza ACN gradient. Total run time 10min.

10µL of the mixture of ABA and KBA in the lower phase of the ATPS 14/14% PEG1000/ ammonium phosphate was injected on HPLC and analysed according to "Ingenza SeQuant ACN gradient" method set (Table 7). The obtained HPLC chromatogram is presented in Figure 18.

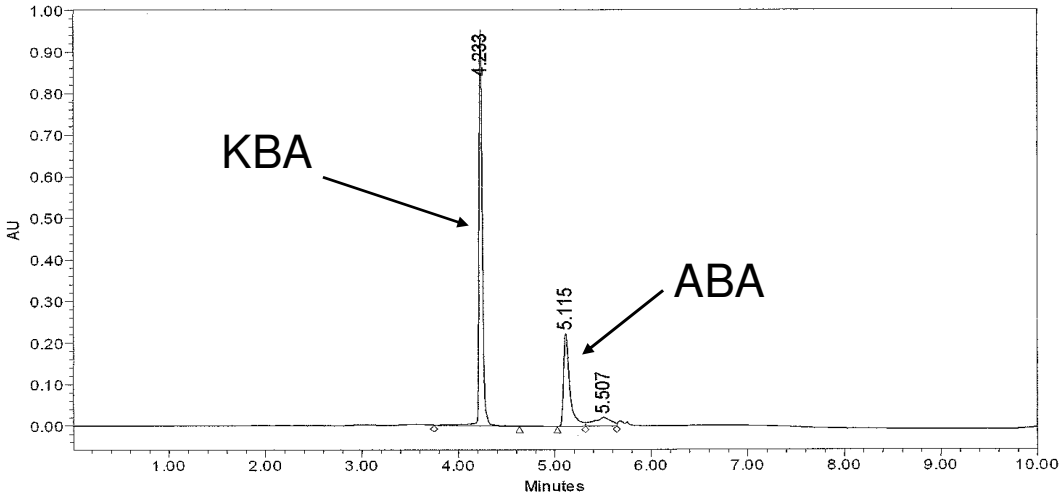


Figure 18 HPLC Chromatogram obtained from the HILIC column according to "Ingenza SeQuant ACN gradient" method set. The ABA and KBA were dissolved in the lower phase of the ATPS 14/14% PEG1000/ammonium phosphate. There are two peaks which elute at 4.2min (KBA) and at 5.1min (ABA). Detection 195nm.

There are two sharp peaks in the chromatogram. Both KBA and ABA elute at different times (4.2min and 5.1min respectively). The presence of the lower phase of the ATPS does not seem to affect the chromatogram. However the HPLC instrument was blocked after two injections. It was presumed that ammonium phosphate, which was a component of the lower phase, precipitated when injected into pure acetonitrile, as the initial condition of the *Ingenza SeQuant ACN gradient* is 100% acetonitrille.

A small test was done and the lower phase formed two phases with ACN when they were mixed together. Moreover there was a white substance in the interface area.

The method was modified to reduce the initial concentration of acetonitrile which precipitated the salt.

After performing an optimisation, the best conditions for the HILIC column method were found to be:

HPLC parameters:

- Injection volume: 10.0μL
- Column temperature: 25°C
- Run time: 7min
- Detector wavelength: 195nm
- Isocratic conditions

	Time [min]	Flow [ml/min]	%A	%B	%C	%D	Gradient
1		1.00	0.0	75.0	0.0	25.0	

Table 8 HPLC conditions to quantify ABA and KBA on the HILIC column. Line B: acetonitrile, Line D: 10mM ammonium formate buffer pH 4.5. Total run time 7min. Method named Ingenza SeQuant Isocratic 8.

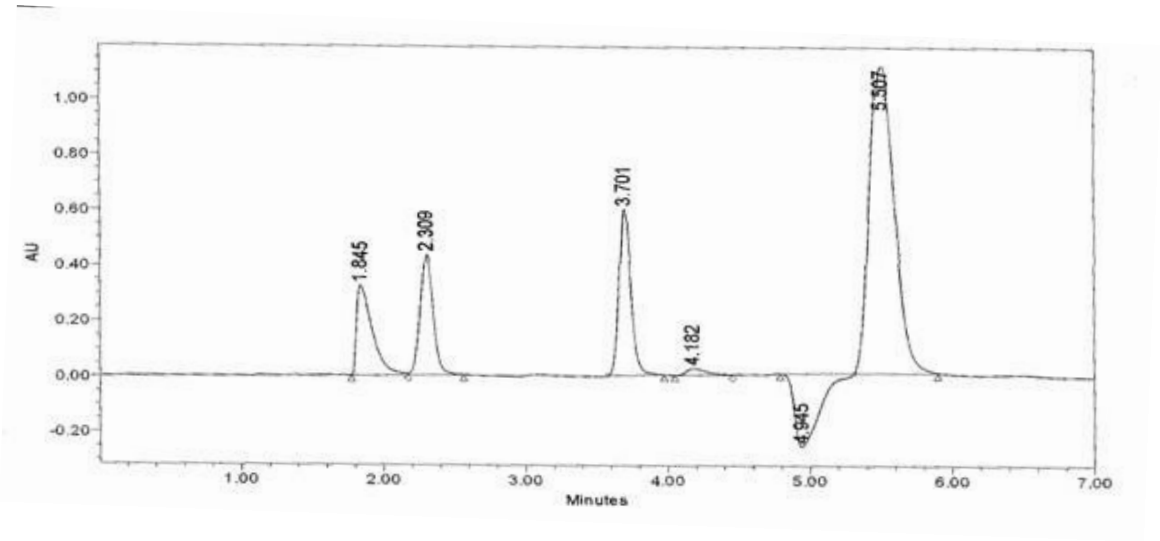


Figure 19 HPLC Chromatogram obtained from the HILIC column according to "Ingenza SeQuant isocratic 8" method set. The ABA and KBA were dissolved in water. Detection 195nm. ABA elutes at 3.7min and 5.5min, KBA at 2.3min.

Figure 19 shows a chromatogram for ABA and KBA analysed on the HILIC column with "Ingenza SeQuant isocratic 8" method set (Table 8). There are four distinct peaks. KBA eluted at 2.3min, whereas ABA is represented by two peaks eluted at 3.7min and 5.5min. There were always the two peaks in the chromatogram regardless of a chiral form of ABA injected, possibly there were two separation methods active within the column. The peak which eluted at 1.8min as well as the negative peak (4.9min) always appeared when water was injected and is therefore likely to be caused by the hydrophilic interaction of water with the column.

Having optimised the method for water (Table 8), its compatibility with ATPS was tested. Peak shape was lost completely, making the chromatogram unreadable when ABA and KBA were dissolved in phase systems made of either PEG3350 or PEG8000. When the two compounds were dissolved in an ATPS made of PEG1000 only ABA eluted as a sharp peak, whereas KBA merged with the neighbouring peak.

Therefore, in spite of the possibility of analysing both ABA and KBA at the same time, the method was not applied for analysis of fractions eluted from CCC.

2.5.1.2. Analysis Method N°2: Aminobutyric acid (ABA) analysis by derivatisation with *ortho*-phthalaldehyde

Aminobutyric acid is a very polar, small organic compound. To quantify it, a derivatisation with *ortho*-phthalaldehyde in the presence of 0.4M potassium borate buffer pH 10 was applied. Figure 20, taken from Roth *et al* (1991)[143], shows a diagram of the derivatisation reaction.

The original method was obtained from Ingenza and modified according to project requirements. The method requires a reducing factor such as 2-mercaptoethanol. Initially this chemical was used, however it does not differentiate the two chiral forms of ABA and a single peak appeared when analysed on C18 HPLC column.

Replacing 2-mercaptoethanol by N-isobutyryl-L-cysteine enabled separation of the two derivatives on a standard C18 column. That was very convenient as the degree of ABA deracemisation could be determined based on one analysis.

The HPLC column used for this method was a C18 GraceSmart RP, particle size 5µm, pore size 18nm, 150mm length and internal diameter 4.6mm.

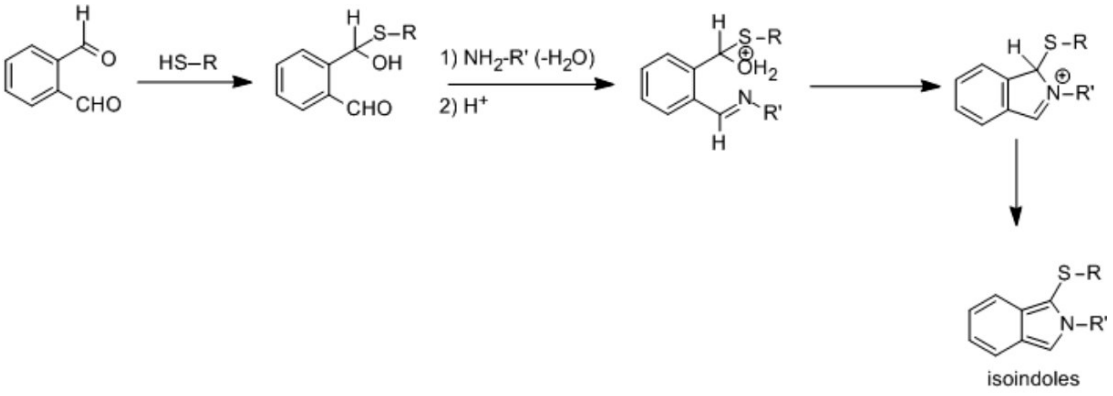


Figure 20. Derivatisation of an amino acid with *ortho*-phthalaldehyde (OPA) in presence of a reducing agent. The derivative can be analysed on HPLC C18 column, detected at 338nm.

a) ABA derivatisation in PEG1000/ammonium sulphate

Initially, ammonium sulphate was chosen as a salt component of ATPS because the presence of ammonium ions was required in the process of KBA reduction to ABA. This process did not involve CCC and was meant to be applied to recycle KBA produced in the CCC-bioreactor.

Using the method received from Ingenza, it was not possible to quantify ABA in the presence of ATPS made of PEG1000/ammonium sulphate, presumably caused by interfering ammonium cations with the derivatisation reagent (*ortho*-phthalaldehyde). The derivatisation method was therefore adapted for the presence of the phase system.

ABA derivatisation in the upper phase

To analyse ABA in the upper phase, the concentration of both *ortho*-phthalaldehyde and 2-mercaptoethanol in a derivatisation solution were increased 2.5 times to have them in excess compared to the ammonium cations. Moreover the volume of analysed sample was reduced by half, thus the ratio of both *ortho*-phthalaldehyde and 2-mercaptoethanol to the sample was increased five times.

The samples were prepared as follows: 50µL of sample was premixed with 100µL of 0.4M potassium borate buffer pH 10 for 30s. Then 100µL of the derivatisation solution (mixture of 67mg *ortho*-phthalaldehyde, 50µL 2-mercaptoethanol, 0.5mL ethanol and 4.5ml 0.4M potassium borate buffer pH 10) was added and incubated for approximately 90s at room temperature. Before injecting on HPLC the sample was diluted by adding 750µL of water.

Upon analysing derivatised samples on HPLC a sudden pressure increase in the column and declining retention time of the compound was noticed. Flushing the HPLC column with methanol revealed a substance accumulated on the HPLC column, presumably created in the side reaction between *ortho*-phthalaldehyde and ammonia. Initially the analysis was performed on a C18 column using an isocratic mode of 25% acetonitrile and 75% potassium phosphate buffer. However, to avoid the accumulation of this ammonium byproduct on the column, a gradient (Table 9) was developed. For each run the acetonitrile concentration was increased to 70%. This prevented the side reaction product from accumulating on the column. The run time was 10min.

	Time [min]	Flow [ml/min]	%A	%B	%C	%D	Gradient
1		1.00	0.0	25.0	0.0	75.0	
2	5.00	1.00	0.0	25.0	0.0	75.0	Linear
3	5.50	1.00	0.0	70.0	0.0	30.0	Linear
4	6.50	1.00	0.0	70.0	0.0	30.0	Linear
5	7.00	1.00	0.0	25.0	0.0	75.0	Linear

Table 9. HPLC method for ABA analysis in the upper phase of PEG1000/ammonium sulphate. Solvent B: acetonitrile, Solvent D: 15mM potassium phosphate buffer pH 7.0. The total run time 10 min. Method named *Ingenza ABA upper phase.*

Figure 21 shows the calibration curve for ABA dissolved in the upper phase of 22/22% PEG1000/ammonium sulphate.

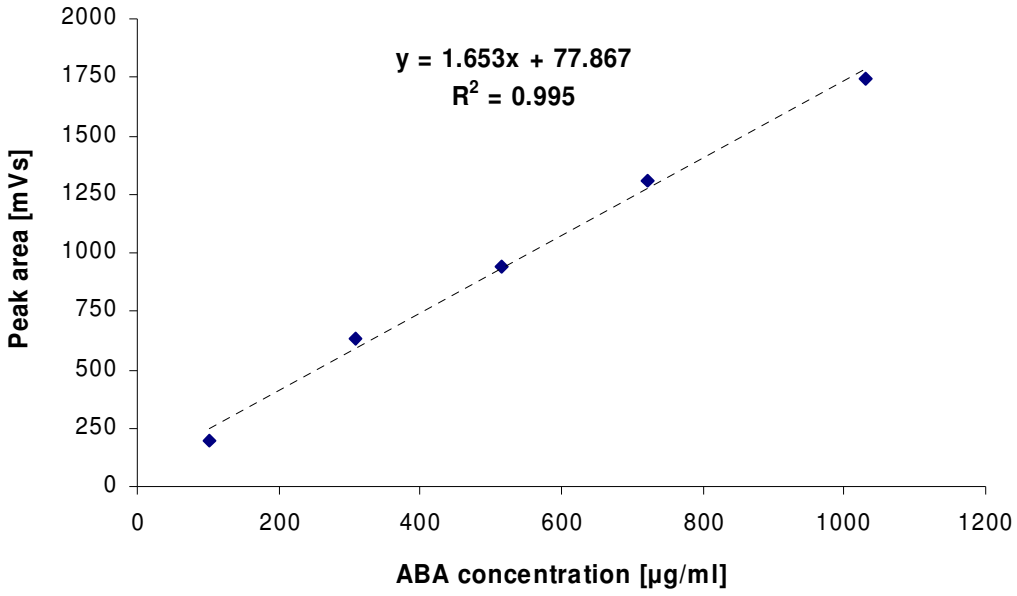


Figure 21 Calibration curve obtained by dissolving ABA in the upper phase of 22/22% PEG1000/ammonium sulphate. Peak area comes from HPLC analysis on C18 column. The method used o-phthalaldehyde and 2-mercaptoethanol and did not differentiate between the ABA enantiomers.

ABA derivatisation in the lower phase

The ABA derivatisation method developed for the upper phase could not be applied for ABA analysis in the lower phase due to the high ammonium ion concentration in the lower, salt-rich phase. To overcome this, the phase was dried and ABA extracted with methanol from the residue.

Procedure description:

100µL of sample was placed into an HPLC vial. The water was evaporated in a centrifugal vacuum concentrator at 60°C. Then 400µL of MeOH was added to the dried sample. ABA is totally soluble in MeOH, whereas ammonium sulphate is not solubilised. To extract ABA trapped in the ammonium sulphate, the sample was sonicated for 5min and mixed on a vortex. Then after the sample was centrifuged to spin down the insoluble ammonium sulphate, 200µL of the methanol supernatant was transferred into a new vial. The MeOH was evaporated and the sample derivatised by dissolving it in 200µL 0.4M potassium borate buffer pH 10 and adding 50µL of the derivatisation solution (27mg *ortho*-phthalaldehyde, 20µL 2-mercaptoethanol, 0.5mL ethanol and 4.5ml borate buffer). The sample was incubated for 2min and diluted with 750µL water before analysis on the HPLC.

In this case, there was no side product of the derivatisation reaction since ammonium sulphate had been removed from the sample. Thus an isocratic analysis method was developed (Table 10) which reduced the run time from 10 to 5 min per sample.

	Time [min]	Flow [ml/min]	%A	%B	%C	%D	Gradient
1		1.00	0.0	30.0	0.0	70.0	

Table 10 HPLC method for ABA analysis in the lower phase of PEG1000/ammonium sulphate. solvent B: acetonitrile, solvent D: 15mM potassium phosphate buffer pH 7.0. Total run time is 5minutes. Method named *Ingenza ABA lower phase.*

Figure 22 shows the calibration curve for ABA dissolved in the lower phase of PEG1000/ammonium sulphate and derivatised according to the ABA derivatisation in the lower phase procedure described above. The curve is linear only within the range of 200-1300µg/ml. Therefore, ABA was only analysed in the lower phase within this range of concentration.

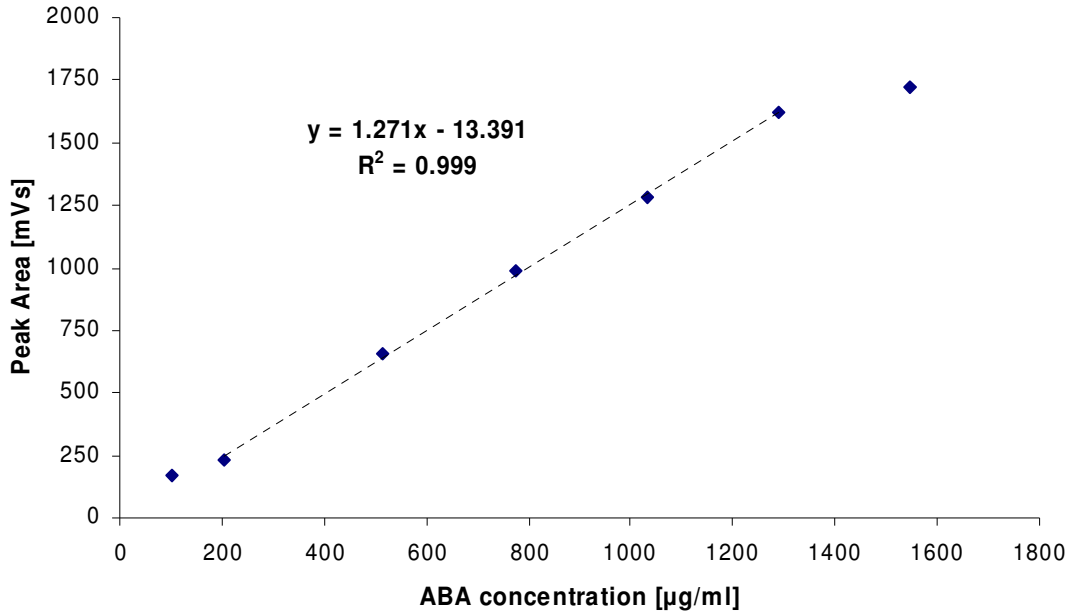


Figure 22 Calibration curve for quantifying ABA in the lower phase of PEG1000/ammonium sulphate using o-phthalaldehyde and 2-mercaptoethanol. Peak area comes from HPLC analysis on C18 column.

b) ABA chiral derivatisation

The chiral derivatisation method was developed by Ingenza. This analytical technique enables quantification of the two ABA enantiomers on a standard C18 HPLC column. The method cannot be used when ABA is quantified in the presence of ammonia salts.

The reagents used were 0.4M potassium borate buffer, pH 10, and a derivatisation reagent made by dissolving 50mg *ortho*-phthalaldehyde and 110mg N-isobutyryl-L-cysteine in 0.5ml MeOH and diluted with 4.5ml borate buffer.

Procedure of the chiral derivatisation:

50µL of an analysed sample was premixed for 30s with 100µL of the borate buffer. Then 100µL of the derivatisation reagent was added and incubated at room temperature for approximately 90s. 750µL of water was then added and the sample analysed by HPLC (C18 column) according to *Ingenza ABA chiral separation* method set (Table 11).

HPLC parameters:

- Injection volume: 10.0µL
- Column type: C18 GraceSmart RP
- Column temperature: 30°C
- Run time: 5min
- Detector wavelength: 338nm
- Isocratic conditions (Table 11)

	Time [min]	Flow [ml/min]	%A	%B	%C	%D	Gradient
1		1.00	0.0	18.0	0.0	82.0	

Table 11 HPLC conditions to separate derivatised D- and L-ABA. Solvent B is acetonitrile, solvent D 15mM potassium phosphate buffer pH 7.0. The method is named as Ingenza ABA chiral separation.

In the chromatogram (Figure 23) there are peaks for L-ABA (2.7min) and D-ABA (3.1min) which were identified by injecting enantiopure standards of D-ABA (CAS 2623-91-3) and L-ABA (CAS 1492-24-6) onto the column. Both standards were purchased from Sigma-Aldrich.

It needs to be noted that the retention times depend on the column and degree of column aging. On the C18 column used in this project, L-ABA always eluted before D-ABA.

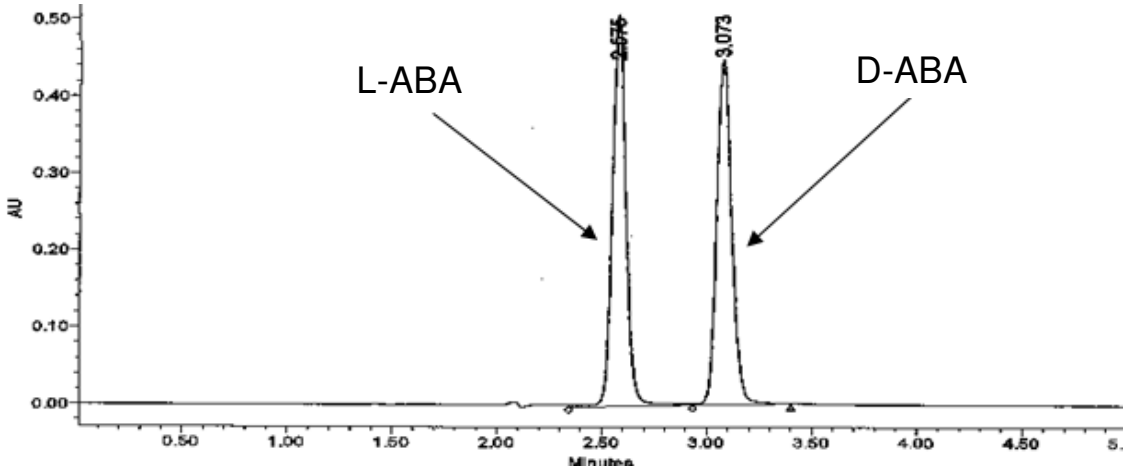


Figure 23 HPLC chromatogram obtained for chiral derivatised D- and L-ABA separated on C18 column. The run time was 5min. Detection 330nm.

Both enantiomers, D-ABA and L-ABA give the same peak area when their racemic mixture was analysed. Thus when an enzymatic conversion by D-amino acid oxidase is performed, L-ABA can be used as an internal standard. It is assumed that initially the peak area of both enantiomers was the same.

Figure 24 shows the calibration curve obtained for ABA. Various dilutions of the ABA stock solution, made by dissolving in water known amounts of D-ABA and L-ABA standards, were derivatised and analysed by HPLC. The two enantiomeric forms of ABA gave the same signal as the two calibration curves totally coincided.

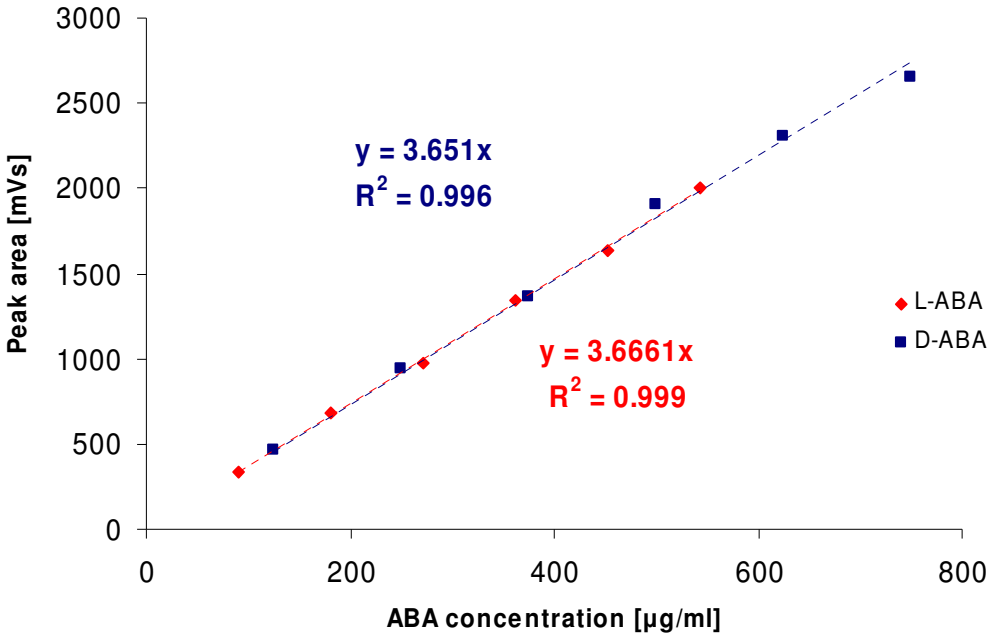


Figure 24 Calibration curves for both D-ABA and L-ABA using a chiral derivatisation method. The two curves totally coincide.

c) ABA chiral derivatisation in various ATPS made of different types of salts

It was not found possible to analyse ABA in the ATPS made of PEG1000 and ammonium sulphate using the ABA derivatisation method. It was suspected that the presence of ammonium ions interacts with the reagents of the derivatisation. To be able to use this analytical method, some other salt, not containing ammonium, had to

be used. For that reason, ABA peak area was determined in 6 ATPS (14/14%) made of PEG 3350 and 6 different salts:

- potassium phosphate, pH 8.0
- potassium citrate, pH 7.6
- sodium phosphate, pH 7.6
- sodium citrate, unbuffered
- potassium sulphate, unbuffered
- sodium sulphate, pH 7.9
- ammonium phosphate, unbuffered

It was not possible to obtain an ATPS 14/14 % PEG 3350/potassium sulphate, as potassium sulphate could not be fully dissolved in presence of PEG 3350. Moreover the ATPS made of 14/14 % PEG3350/ammonium phosphate was also tested to prove that the presence of ammonium adversely affects results.

50µL solution including 1.11mg D/L-ABA was dissolved in 4950µL of each phase or water for the control. Next 100µL of each sample was derivatised for ABA and analysed on HPLC. Results are presented in Table 12.

ATPS 14/14 % PEG3350/Salt			
Salt type	**Phase UP/LP**	**Peak Area [mVs]**	
		L-ABA	**D-ABA**
Potassium Phosphate	**UP**	412	418
	LP	409	415
Potassium Citrate	**UP**	404	408
	LP	411	419
Sodium Phosphate	**UP**	415	422
	LP	403	410
Sodium Citrate	**UP**	411	416
	LP	410	416
Sodium Sulphate	**UP**	412	417
	LP	416	420
Ammonium phosphate	**UP**	125	124
	LP	-	-
Potassium sulphate	**Salt was not soluble in the presence of PEG3350**		
Water	**-**	413	419

Table 12 HPLC analysis of D-ABA and L-ABA by chiral derivatisation (2.5.1.2b) in 6 different ATPS (14/14% w/w) PEG3350 and a salt. The peak area is given separately for D-ABA and L-ABA for each phase. UP stands for upper phase, whereas LP for lower phase.

Apart from the ATPS made of ammonium phosphate, the peak area for both D- and L-ABA was approximately the same in all the tested phases and water (control). No peak was obtained when the lower phase of the ATPS made of ammonium phosphate was tested. Also the area obtained for the upper phase of that system was significantly smaller than the control sample (water) as well as other salts. Thus the presented ABA chiral derivatisation can be used in presence of all the tested salts apart from ammonium phosphate.

d) Stopping the enzymatic reaction by chiral derivatisation of ABA

The most accurate method for the determination of enzyme activity seemed to be quantification of either the increase of the product created by catalytic action or the decrease of the converted substrate. The enzymatic reaction had to be completely quenched at a required time to ensure there was no further substrate conversion before a sample was analysed on HPLC. In the following experiment, it was investigated if adding the derivatisation reagent quenches the enzymatic reaction.

According to the procedure received from Ingenza (*Procedure of chiral derivatisation*, section 2.5.1.2b), ABA derivatisation consists of two steps:

1. premixing the sample with borate buffer pH 10 for 30s.
2. addition of the derivatising reagent to the sample and incubating it for 90s.

In the first experiment it was investigated if the presence of borate buffer, pH 10, stops the enzymatic conversion of D-ABA. 3.4mg DL-ABA was placed in 1.9ml of 50mM potassium phosphate buffer pH 7.5. 100µL of the DAAO supernatant was added to start the enzymatic conversion. 1ml of this solution was immediately mixed with 1ml of 0.4M borate buffer pH 10. Every 2min 200µL of the solution was derivatised with 50µL of the chiral derivatising reagent and analysed on HPLC.

Table 13 shows that the enzymatic reaction was only partially inhibited when a borate buffer, pH 10, was added to a sample, which is the first step of the derivatisation procedure. By contrast, the control sample of DL-ABA incubated with DAAO for 60min gave a ratio of D-ABA/L-ABA of nearly zero.

	Derivatisation time [min]	Peak area [mVs]		Ratio D/L
		L-ABA	D-ABA	
Borate buffer pH 10 added	2	2977	2524	**0.85**
	4	3001	2443	**0.81**
	6	3011	2365	**0.79**
	8	3011	2279	**0.76**
	10	2981	2096	**0.70**
	60	2970	1380	**0.46**
Control	60	3027	20	**0.01**

Table 13 HPLC analysis of D-ABA converted by DAAO in presence of borate buffer pH 10

Since the addition of borate buffer, pH 10, to the sample did not stop the enzymatic reaction, it was investigated if the reaction would stop after the addition of the derivatisation reagent. 3.4mg DL-ABA was dissolved in 1.9ml of 50mM potassium phosphate buffer pH 7.5. 100µL of the DAAO supernatant was added to start the enzymatic conversion. 50µL of this solution was immediately derivatised with the derivatising reagent premixed with the borate buffer. The rest of the solution was incubated in room temperature to complete the enzymatic reaction (control). The derivatised sample was injected every 5min on HPLC (repetitive injections).

It has been found out that the chiral ABA derivatisation stops the enzymatic conversion of D-ABA by D-amino acid oxidase (Table 14). The ratio D- to L-ABA was constant (0.93) in all 10 injections from 0 to 70min, demonstrating that the action of DAAO had been stopped by adding the derivatisation reagents. By contrast the control sample of DL-ABA incubated with DAAO for 60min gave a ratio of D-ABA/L-ABA of zero.

	Time [min]	Peak area [mVs]		Ratio D/L
		L-ABA	D-ABA	
Sample taken at the beginning of the enzymatic reaction derivatised and injected 10 times on the HPLC	0	3032	2825	**0.93**
	5	3039	2819	**0.93**
	10	3027	2826	**0.93**
	15	3044	2827	**0.93**
	20	3046	2837	**0.93**
	25	3051	2833	**0.93**
	30	3037	2814	**0.93**
	35	3060	2844	**0.93**
	40	3029	2825	**0.93**
	45	3027	2823	**0.93**
	70	3029	2820	**0.93**
Control	60	3022	14	**0.00**

Table 14 Demonstration that the chiral derivatisation method stops the enzymatic action of DAAO in D-ABA. HPLC peak area of D-ABA and L-ABA obtained for the derivatised enzymatic solution.

Since it would be difficult to predict how much of a substrate was converted in the first step, which involves the addition of the borate buffer, a sample needed to be derivatised in a single step with the mixture of the derivatising reagent and the borate buffer, pH 10.

It was unknown how elimination of the first step affected the ABA quantification. This was investigated in the following experiment.

The ABA stock solution was derivatised in a single step by adding a mixture of the derivatising reagent and the borate buffer. See paragraph above, (*Procedure for the derivatisation stopping the enzymatic reaction*). This was compared to a control when the two steps were done according to the procedure given by Ingenza. See *Procedure of the chiral derivatisation* (section 2.5.1.2b). Results are presented in Table 15

Replication	Method 1			Method 2		
	Sample premixed with the buffer and then derivatised			Sample derivatised with the derivatisation solution premixed with the buffer		
	Peak area [mVs]		Ratio L/D	Peak area [mVs]		Ratio L/D
	L-ABA	D-ABA		L-ABA	D-ABA	
1	6541	6755	0.968	6270	6371	0.984
2	6427	6562	0.979	6318	6446	0.980
3	6425	6556	0.980	6251	6367	0.982
Mean	6464	6624	**0.98**	6279	6395	**0.98**
St dev	66	113	**0.007**	34	44	**0.002**
St. Dev %	1.03	1.71	**0.68**	0.55	0.69	**0.21**

Table 15 Comparison between the two ABA chiral derivatisation methods. Method 1: 30s incubation of a sample with a borate buffer prior to derivatisation. Method 2: the sample is derivatised with a mixture of the derivatisation solution and a borate buffer.

Premixing with the buffer (Method 1) gave a slightly bigger peak area (3%). That is considered more sensitive, as more ABA was derivatised and thus detected on HPLC. However, there is a slightly bigger variation between the three replicates, which is probably caused by there being two pipetting steps: first buffer was added and then the derivatisation solution. The ratio D- to L-ABA was the same for the both tested methods. There was no significant difference between the two groups (P value equals 0.20).

The chiral derivatisation in a single step (2.5.1.2d) was used to determine the enzyme activity and stability as well as ABA eluted from the CCC-bioreactor. When more accurate ABA quantification was needed (e.g. to determine the D-value), the two-step derivatisation (2.5.1.2b) was performed.

For experiments which involved stopping the enzymatic reaction by ABA derivatisation, a modified procedure, which is described below, was established.

Procedure for the derivatisation stopping the enzymatic reaction:

50µL sample was mixed with 150µL of the derivatising reagent (50mg *ortho*-phthalaldehyde and 110mg N-Isobutyryl-L-cysteine dissolved in 0.5ml MeOH, diluted to 15ml with 0.4M borate buffer pH 10), and incubated for 2min. It was then diluted with 800µL water and analysed on an HPLC C18 column (Table 11).

2.5.1.3. Analysis Method N°3: Ketobutyric acid (KBA) analysis on an Anion Exchange Column

KBA is a product of the enzymatic action of the DAAO on D-ABA. Three HPLC methods were developed for KBA analysis. The method which used the HILIC column allowed detection of both KBA and ABA and was presented in section 2.5.1.1. Other two methods are: direct analysis on an anion exchange column (this section) and derivatisation with phenyl hydrazine (section 2.5.1.4).

This method, which was given by Ingenza, allows for the direct quantification of KBA. It has been successfully applied to the analysis of KBA in a phase system. The biggest advantage of this method is its simplicity as no derivatisation is required. Thus an analysis sample can be directly injected on HPLC. However, the method is not as sensitive as the derivatisation method described in the next section (2.5.1.4).

The method involves a SphereClone 5µm SAX ion exchange column which was purchased from Phenomenex. The column length was 150mm and the internal diameter 4.6mm.

HPLC parameters:

- Injection volume: 10.0µL
- Column temperature: 40°C
- Run time: 15min

- Detector wavelength: 220nm
- Gradient conditions given in Table 16

	Time [min]	Flow [ml/min]	%A	%B	%C	%D	Gradient
1		1.50	90.0	0.0	0.0	10.0	
2	0.10	1.50	90.0	0.0	0.0	10.0	Linear
3	10.00	1.50	0.0	0.0	0.0	100.0	Linear
4	12.00	1.50	0.0	0.0	0.0	100.0	Linear
5	13.00	1.50	90.0	0.0	0.0	10.0	Linear

Table 16 HPLC Ion Exchange method to quantify KBA. Line A was water and Line D: 10mM ammonium phosphate buffer pH 8.0. SphereClone 5µm SAX Ion Exchange column. Method named *Ingenza KBA IonExchange.*

The peak elutes after about 8 minutes (Figure 25). The method allows analysis of KBA in both phases of the ATPS.

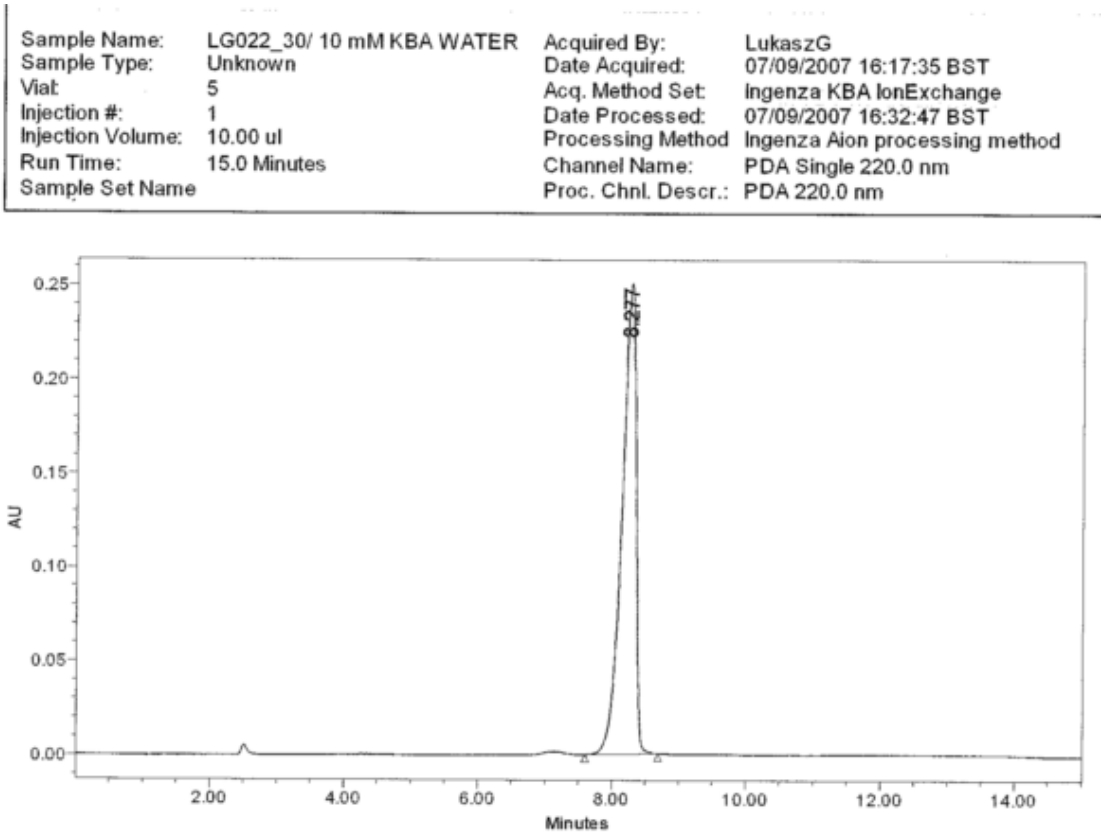


Figure 25 HPLC Chromatogram obtained for KBA analysis with the anion exchange column according to "Ingenza KBA Ion Exchange" method set detailed in Table 16.

A KBA calibration curve was performed on HPLC using the anion exchange column (Figure 26). The KBA solutions (126-2627µg/ml) were made in water. It was experimentally confirmed that the presence of a phase system of PEG1000/ammonium sulphate does not affect of the KBA peak area.

Despite its easy use and convenience, the method has some disadvantages. The column is expensive and its lifetime is relatively short. It allows for analysis of about 100 samples only. That creates high costs, especially considering that a single CCC experiment generates about 20 fractions. Thus one column can be used for 5-6 sample sets before requiring replacement. Moreover the equation of the calibration curve obtained from the sample analysis on the anion exchange column was y=3787x (Figure 26), whereas for the next method, which was analysing a sample derivatised with phenyl hydrazine on a standard C18 column, y=8054x (Figure 32). Therefore this method is only half as sensitive as the second method, which is presented below (2.5.1.4).

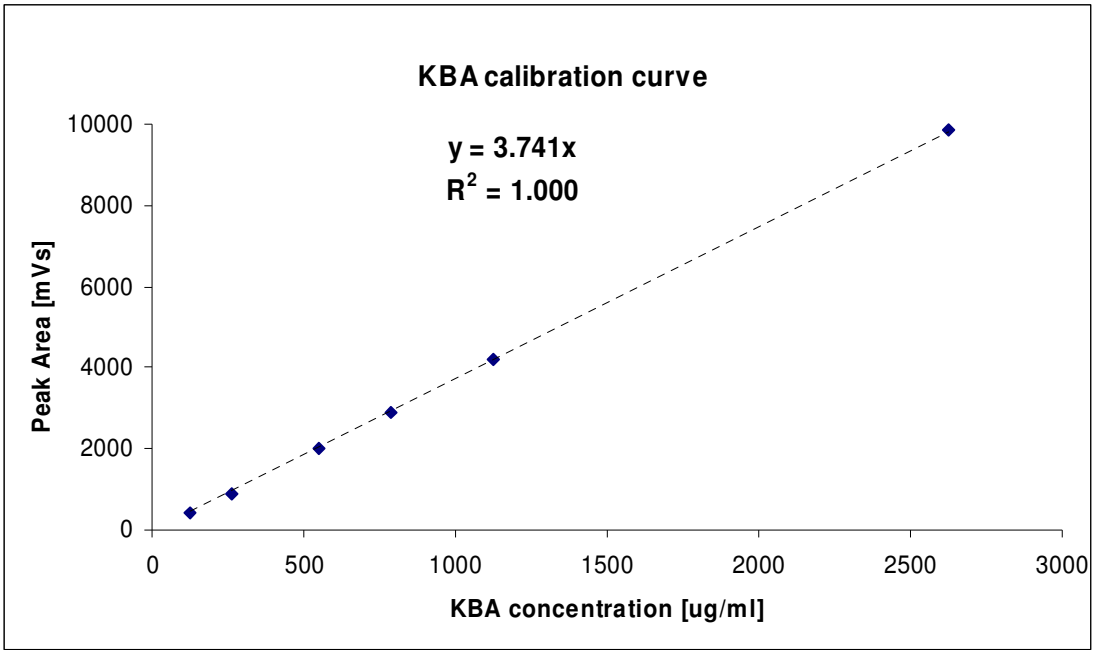


Figure 26 Calibration curve for KBA in water obtained on the anion exchange HPLC column

2.5.1.4. Analysis Method N°4: KBA derivatisation with phenyl hydrazine

The method is based upon the KBA derivatisation with phenyl hydrazine in the presence of 150mM potassium phosphate buffer, pH 8 (Figure 27).

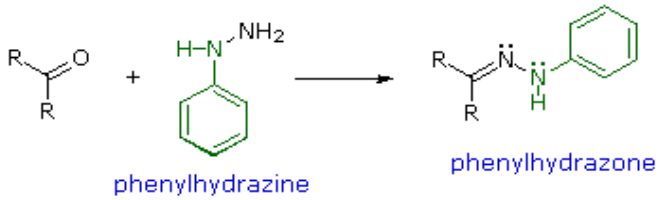


Figure 27 Derivatisation of a keto group with phenyl hydrazine. The derivative (phenylhydrazone) can be analysed on a C18 HPLC column.

It is known that hydrazones derived from ketones are present as mixtures of two isomers in equilibrium[144] (Figure 28). Pellegrino *et al*[145] using a liquid chromatography column (RP-18) resolved the two isomers of phenylhydrazones derived from phenylhydrazine and acetaldehyde.

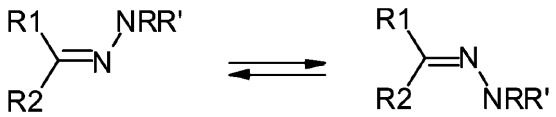


Figure 28 The two isomers for hydrazones derived from ketones[144].

Figure 29 shows two isomers of phenylhydrazone derived from phenylhydrazine and 2-keto butyric acid. It is possible that the *cis*-isomer forms a stable 6-membered ring structure (Figure 30) which is less polar and therefore should elute after a longer time.

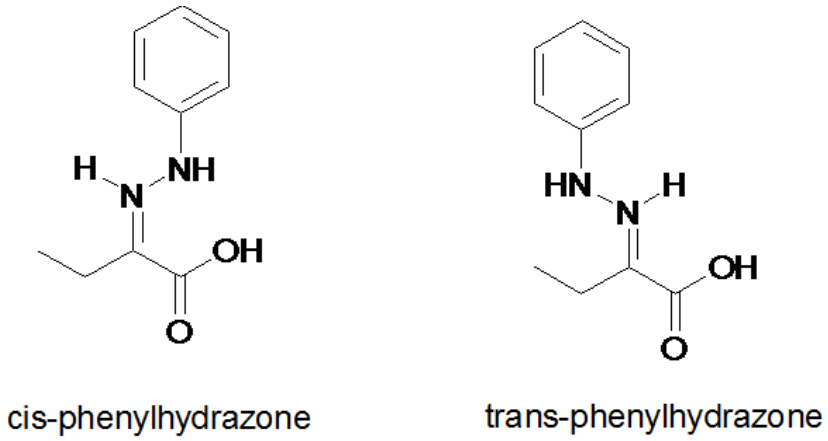


Figure 29 Two isomers of phenylhydrazone derived from ketobutyric acid (KBA).

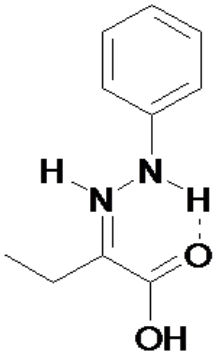


Figure 30 Possible internal hydrogen bond in *cis*-isomer of phenylhydrazone.

The HPLC method, suggested by Ingenza, was modified by increasing the concentration of methanol in the mobile phase from 7 to 35%. This allowed the second KBA derivative to elute. The high concentration of methanol in the mobile phase affected the resolution between eluted compounds. That was improved by decreasing the flow rate of the mobile phase from 1 to 0.8ml/min. The HPLC conditions used for analysis of KBA are presented in Table 17.

Derivatisation procedure:

200µL of a sample was mixed with 400µL 150mM potassium phosphate buffer pH 8.0 and 400µL 0.05M phenylhydrazine solution. The incubation time with a derivatising reagent is at least 20min at room temperature.

HPLC parameters:

- Injection volume: 10.0µL
- Column type: C18 GraceSmart RP
- Column temperature: 40°C
- Run time: 8min
- Detector wavelength: 324nm
- Isocratic conditions

	Time [min]	Flow [ml/min]	%A	%B	%C	%D	Gradient
1		0.80	0.0	0.0	35.0	65.0	

Table 17 HPLC conditions to quantify derivatised KBA. Solvent C is methanol, solvent D 15mM potassium phosphate buffer, pH 8.0. The method is named as Ingenza KBA C18.

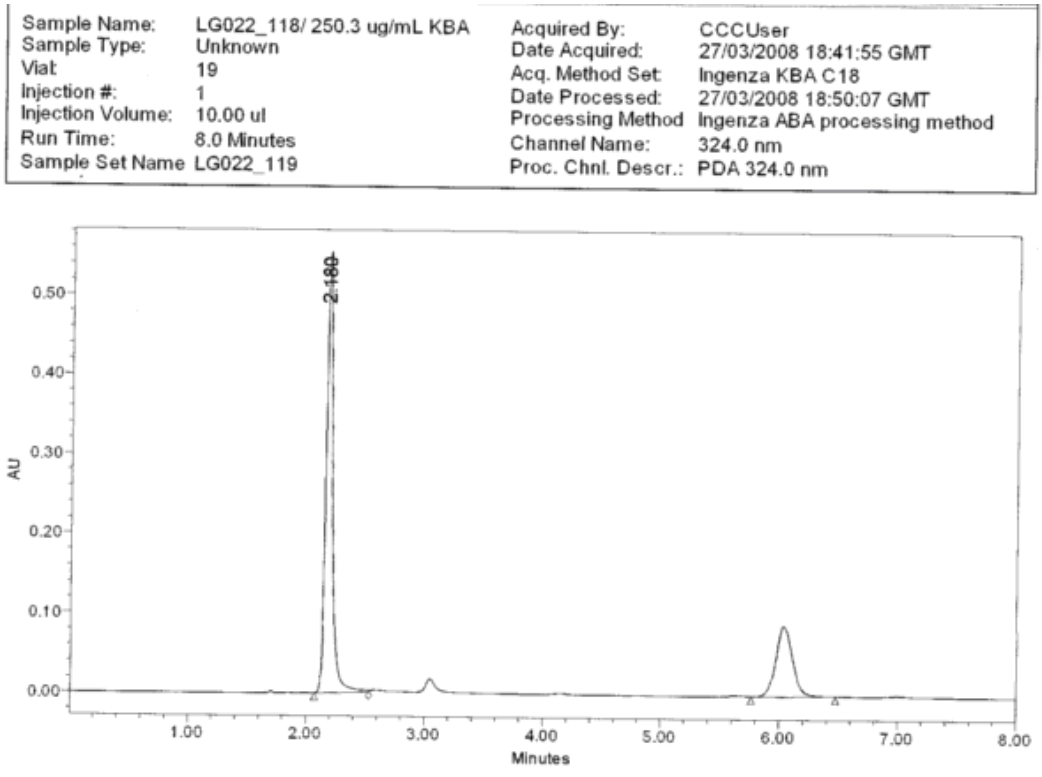


Figure 31 HPLC Chromatogram obtained for KBA derivatised with phenyl hydrazine, analysed on the C18 column according to "Ingenza KBA C18" method set (Table 17). There are two peaks for KBA, at 2.1min and 6min.

In the chromatogram (Figure 31) there are two peaks which elute at 2.1min and 6min. Both of them are proportional to the KBA concentration in a sample. The first peak might be the more polar *trans*-form of KBA-derivative, whereas the second the less polar *cis*-form.

The area of the first peak (Figure 31), which elutes at 2.1min, is always 2.5 times bigger than area of the second peak. Moreover, it is sharper which makes peak integration more accurate. Therefore to increase sensitivity, all KBA analysis was performed based on the first peak. Figure 32 shows the calibration curve for KBA based on the first eluted peak.

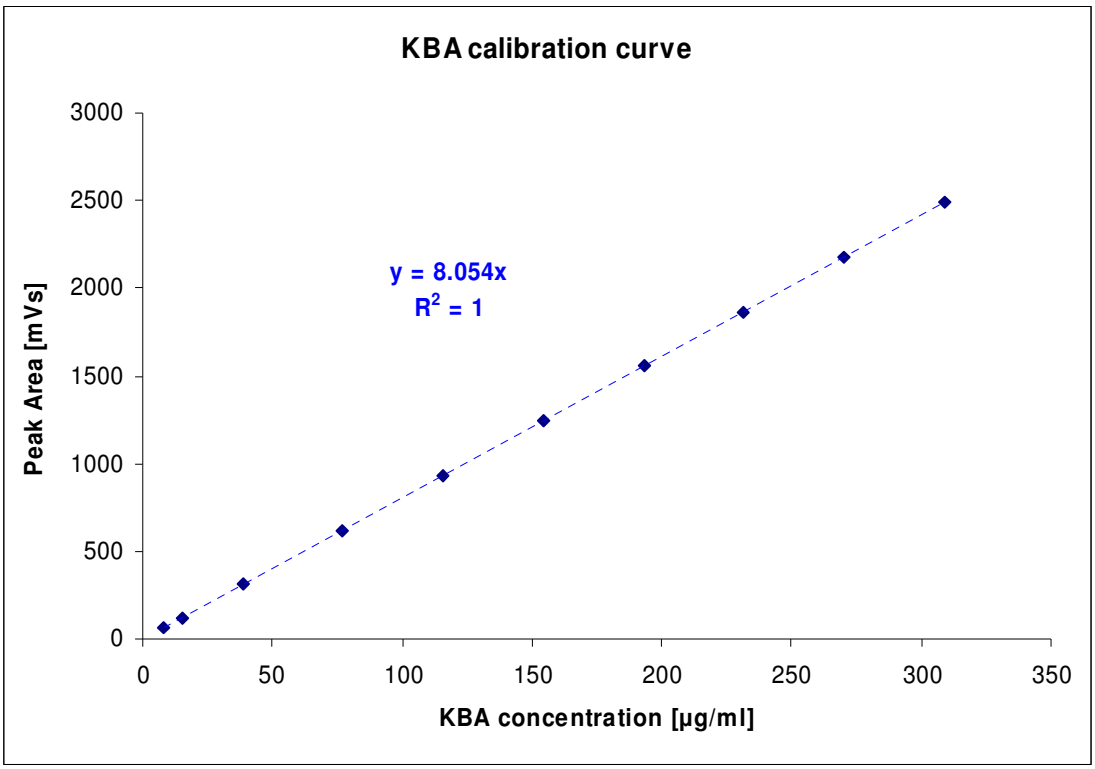


Figure 32 Calibration curve for KBA based on HPLC analyses. The area of the first peak which eluted at about 2min plotted against KBA concentration.

It was confirmed that KBA can be quantified in an ATPS based on ammonium sulphate salt. 1ml of KBA stock solution (1.5mg/ml) was placed into 9.0ml of water (control), 9.0ml of the upper phase and 9.0ml the lower phase of ATPS (PEG1000/ammonium sulphate). Samples were derivatised with phenyl hydrazine and analysed by HPLC. Results are presented in Table 18. In all 3 cases the difference in the peak areas were within 5% of each other and the values were within 5% of the area expected from the calibration curve (Figure 32).

	Peak area [mVs]	Calculated KBA concentr. [µg/ml]
Water	**1224**	**152**
Upper phase	**1273**	**158**
Lower phase	**1184**	**147**
Mean	1227	152.3
SD	44	5.51
CV [%]	3.62	3.62

Table 18 HPLC analysis of KBA in a phase system of ATPS PEG1000/ammonium sulphate. SD – standard deviation of the three values. CV- coefficient of variation calculated by dividing SD by the mean and multiplied by 100%.

Injecting the same sample six times over a period of 3 hours shows that the derivative (2.1min) is stable for several hours (Figure 33).

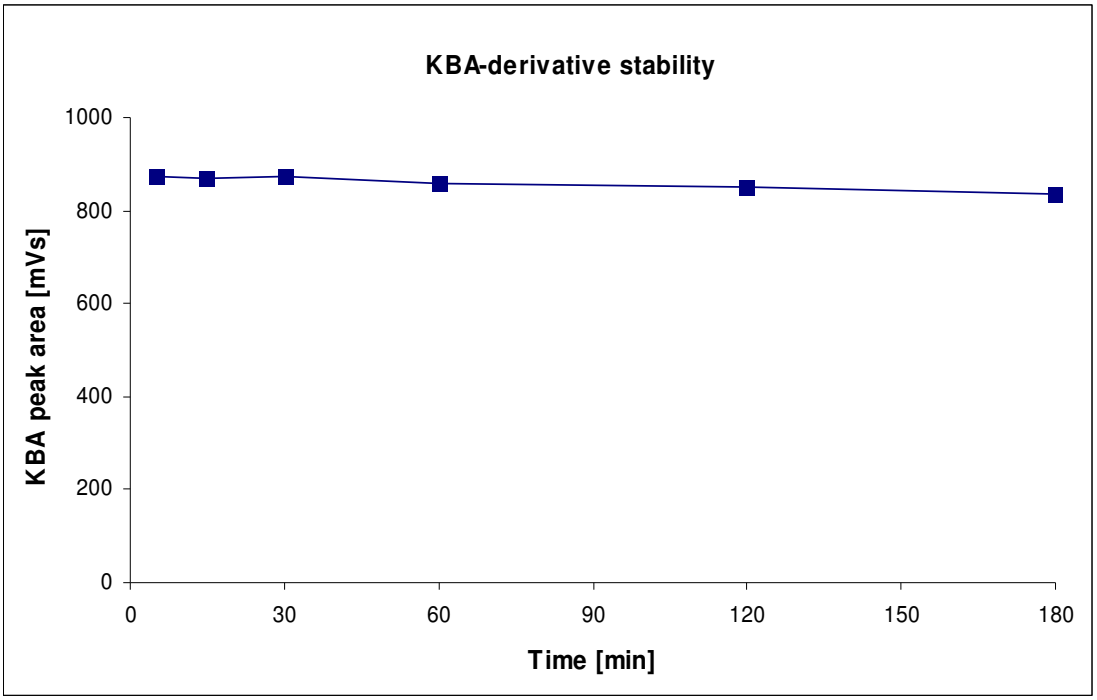


Figure 33 Stability of KBA-derivative (2.1min) determined on HPLC. The derivatised KBA solution was injected 6 times on HPLC. For the sample injected 3h after the derivatisation the peak area was 4% smaller compared to the same sample injected 5min after the derivatisation.

2.5.2. Enzyme activity determination

Finding a phase system which did not inactivate the enzyme was found to be considerably more difficult then first expected (Chapter 3). For that reason an analytical method which enabled determination of enzyme activity in a phase system was required.

Two methods were developed: N°1 a colorimetric enzyme assay (section 2.5.2.1) and N°2 monitoring enzyme activity based on the direct HPLC analysis of the converted substrate (section 2.5.2.2.).

2.5.2.1. Enzyme activity Method N°1 - assay based on the colorimetric reaction

Materials used for the colorimetric enzyme assay

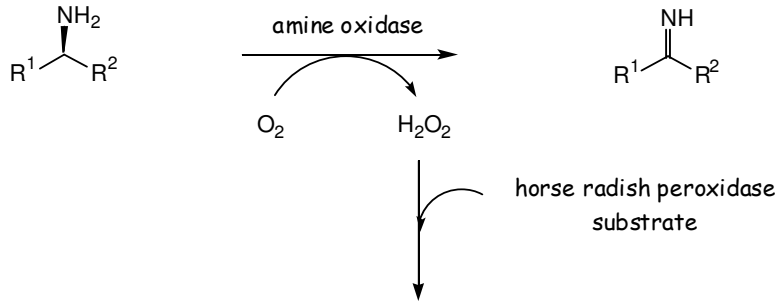
Name	Abbreviation	CAS	Supplier
3-Hydroxy-2,4,6-tribromobenzoic acid	TBHBA	14348-40-4	Alfa Aesar
4-Aminoantipyrine	4-AAP	83-07-8	Sigma
Dimethyl Sulphoxide	DMSO	67-68-5	Fisher Chemical
Peroxidase (horseradish roots)	HRP	9003-99-0	Sigma

Table 19 Suppliers of the reagents used for the colorimetric assay of D-amino acid oxidase (DAAO).

General assay description

The assay was provided by Ingenza and modified to make it suitable for the current project and available equipment.

The colorimetric assay relies on quantification of hydrogen peroxide (H_2O_2), the by-product of the oxidase reaction. The hydrogen peroxide is reacted upon by the second enzyme, horseradish peroxidase (HRP), which oxidases the co-substrate, 4-aminoantipyrine/ 2,4,6-tribromo-3-hydroxybenzoic acid (4-AAP/TBHBA), generating a coloured product. Since the assay is based upon the production of H_2O_2, it can be used for testing the activity of any amine oxidase that generates H_2O_2.



Pink colour (detected at 490nm)

Figure 34 Diagram shows the principle of the colorimetric reaction used to determine the activity of an amino acid oxidase

The assay was mainly used to determine the activity of the stored enzyme as well as its stability in ATPS.

Reagent preparation:

Stock solution of assay mix:

10ml 1M potassium phosphate buffer, pH 7.8

39ml water

20mg TBHBA in 1ml DMSO

15.2mg 4-AAP

Assay solution:

5ml stock solution of assay mix

0.0104g D-ABA

4.4ml water

100µL HRP solution (7750units/ml)

The assay solution can be stored in a freezer for a few months provided that it does not contain HRP. HRP can be added just before analysis, after thawing.

Assay performance

The assay was performed on a 96 well plate. A 10μL sample was placed into each well (in triplicate). Next, by means of a 12 channel pipette, 190μL of the assay solution was added to each well and mixed with a pipette. The intensity of the colour product was measured at 490nm using a 96 well plate reader. The absorbance was measured 4-5 times at 1.5 or 2 minute intervals.

The enzyme activity was proportional to the slope obtained by plotting in Excel the absorbance against the time [min] of taken measurements.

Despite the simplicity and convenience, the assay has some features which limit its application in phases containing organic solvents, as those could interact with the second enzyme (HRP), giving a false result. Moreover an assayed sample, which potentially is a tested phase system, is diluted 20 times with the assay solution. This contains 0.1M phosphate buffer, pH 7.8, (see below) which creates an optimal environment for the enzyme. During an assay the enzyme would work in the buffer solution instead of a tested phase system. Therefore to test activity of the enzyme in a phase system, HPLC analysis of ABA (section 2.5.2.2) was used.

2.5.2.2. Enzyme activity method N°2 - enzyme activity based on a direct D-ABA conversion

The enzyme activity is proportional to the % of deracemised ABA in a given time. To determine the enzyme activity, the enzyme was incubated with a racemic mixture of ABA for a given time and derivatised for ABA, which stopped the enzymatic reaction. Then the sample was analysed on HPLC. L-ABA was used as an internal standard and it was assumed that initial the D- and L-ABA concentrations in a sample were the same. Thus the difference between D-ABA and L-ABA peak area, after the incubation with the enzyme, is proportional to the enzyme activity.

This direct assay is more suitable to determine enzymatic activity in a phase system compared to the colorimetric assay as the conversion of D-ABA is monitored directly.

2.5.3. Enzyme stability determination

The enzyme stability was determined by comparing the enzyme's activity after a certain time of incubation in a phase system with its initial activity in that phase. To measure the enzyme activity, both methods were used i.e. the colorimetric assay N°1 (2.5.2.1) and monitoring D-ABA conversion on HPLC N°2 (2.5.2.2).

2.5.3.1. Determination of the enzyme stability by the colorimetric assay (N°1)

Description of the method:

2ml of a phase system was placed into a polypropylene tube with a snap cap (1ml upper phase and 1ml lower phase). Next 50µL of the enzyme solution was added, mixed vigorously with a phase system and 10µL immediately transferred into the well of a 96 well plate before the two phases started separating from each other. This was done in triplicate so each of three wells was filled with 10µL of the same phase system. The assay for the enzyme activity (2.5.2.1) was then performed and the activity was recorded as the initial activity. The tubes with the tested systems were placed on a rotating wheel so they were continuously mixed, to avoid phase separation as well as to simulate conditions in CCC where the enzyme would stay in contact with both phases. The samples were analysed again after another 2 and 4 hours.

The enzyme stability was expressed as % of the activity after 2 and 4 hours compared to the initial activity, which was assumed to be 100%.

2.5.3.2. Determination of the enzyme stability by the direct HPLC analysis of converted substrate (N°2)

1ml of each phase of a phase system was placed into a polypropylene tube with a snap cap. Next 50µL of the enzyme solution was added and the tube with the phase system was placed onto a rotating wheel so the two phases were continuously mixed. After 2 or 4 hours the substrate, DL-ABA was added and the tube was placed back onto the rotating wheel. The enzymatic reaction was continued for 10 min and stopped by derivatising 50µL of sample with the solution of *ortho*-phthalaldehyde and N-isobutyryl-L-cysteine which stopped the enzymatic reaction. The % of converted D-ABA was calculated based on HPLC analysis.

For the control, first the substrate solution was added to the phase system, then the enzyme. The reaction was carried on for 10min and the sample was derivatised with *ortho*-phthalaldehyde N-isobutyryl-L-cysteine and analysed on the HPLC.
The enzyme stability was determined based on the % of D-ABA converted by the enzyme incubated for 2 or 4 hours in a phase system compared to % the of D-ABA converted by the fresh enzyme (initial activity).

2.5.4. Enzyme concentration determination

To determine an enzyme protein concentration in a sample, a modified Bradford method was used. The Bradford assay involves the binding of Coomassie Brilliant Blue G-250 to protein, which causes a shift of the absorption maximum of the dye from 365 to 595nm. The increase of absorption at 595nm is monitored[146]. The method is based on the observation that Coomassie Brilliant Blue G-250 exists in two different colour forms, red and blue. The red form is converted into blue upon binding the dye to protein. The complex is formed within 2min and is stable for about 1 hour.

Zor and Selinger[147] (1996) demonstrated the linearity of the ratio of absorbance 590nm over 450nm with protein concentration. Protein determination by measuring A_{590}/A_{450} ratio is more accurate than a single measurement of A_{590}, giving about a 10-fold increase in accuracy and sensitivity compared to the standard Bradford assay.

The method was adapted for determination of enzyme protein concentration. The colorimetric reaction was done in a 96 well plate and the absorbance was read after 10 min on a plate reader (Biohit model).

5 solutions of albumin (BSA, Sigma-Aldrich, CAS 9048-46-8) were made at different concentrations (0.2-1.0mg/mL). 10µL of each concentration was placed into the wells of a 96 well plate in triplicate. Then 290µL of Bradford reagent (Sigma-Aldrich, cat No B6916), which contained Coomassie dye, was added to each well. After 10min the absorbance was read at 450, 600 and 630nm on the plate reader. The plate reader used was factory-equipped with 4 filters which allowed measurement of absorbance at 405, 450, 490 and 630nm. An extra 600nm filter was available only for the time of the experiment.

Results are presented in the Figure 35. A higher sensitivity (greater slope), was obtained when the ratio of the two measurements (A_{600}/A_{450} and A_{630}/A_{450}) rather than single measurements (A_{600} and A_{630}) were plotted against the protein concentration.

This agrees with the report of Zor and Selinger [147]. Also measuring the absorbance at 600nm rather than at 630nm gave a better sensitivity.

The slopes (absorbance/concentration) obtained for A_{600}/A_{450} and A_{630}/A_{450} were 0.99 and 0.84 respectively. However, for the subsequent protein analysis the ratio of A_{630}/A_{450} was used.

Albumin concentration [mg/ml]	Absorbance at 600 nm					Absorbance at 450 nm				Ratio 600/450				
	Rep 1	Rep 2	Rep 3	Mean	St. error	Rep 1	Rep 2	Rep 3	Mean	Rep 1	Rep 2	Rep 3	Mean	St error
1.0	0.996	1.052	1.121	**1.056**	**0.036**	0.698	0.735	0.816	**0.750**	1.427	1.431	1.374	**1.411**	**0.019**
0.8	0.868	0.926	0.938	**0.911**	**0.022**	0.755	0.753	0.741	**0.750**	1.150	1.230	1.266	**1.215**	**0.034**
0.6	0.786	0.804	0.813	**0.801**	**0.008**	0.805	0.791	0.783	**0.793**	0.976	1.016	1.038	**1.010**	**0.018**
0.4	0.686	0.686	0.69	**0.687**	**0.001**	0.841	0.847	0.833	**0.840**	0.816	0.810	0.828	**0.818**	**0.005**
0.2	0.548	0.571	0.563	**0.561**	**0.007**	0.897	0.925	0.902	**0.908**	0.611	0.617	0.624	**0.617**	**0.004**

Albumin concentration [mg/ml]	Absorbance at 630 nm					Absorbance at 450 nm				Ratio 630/450				
	Rep 1	Rep 2	Rep 3	Mean	St. error	Rep 1	Rep 2	Rep 3	Mean	Rep 1	Rep 2	Rep 3	Mean	St error
1.0	0.923	0.979	1.045	**0.982**	**0.035**	0.698	0.735	0.816	**0.750**	1.322	1.332	1.281	**1.312**	**0.016**
0.8	0.816	0.866	0.875	**0.852**	**0.018**	0.755	0.753	0.741	**0.750**	1.081	1.150	1.181	**1.137**	**0.030**
0.6	0.754	0.769	0.775	**0.766**	**0.006**	0.805	0.791	0.783	**0.793**	0.937	0.972	0.990	**0.966**	**0.016**
0.4	0.673	0.677	0.679	**0.676**	**0.002**	0.841	0.847	0.833	**0.840**	0.800	0.799	0.815	**0.805**	**0.005**
0.2	0.564	0.585	0.581	**0.577**	**0.006**	0.897	0.925	0.902	**0.908**	0.629	0.632	0.644	**0.635**	**0.005**

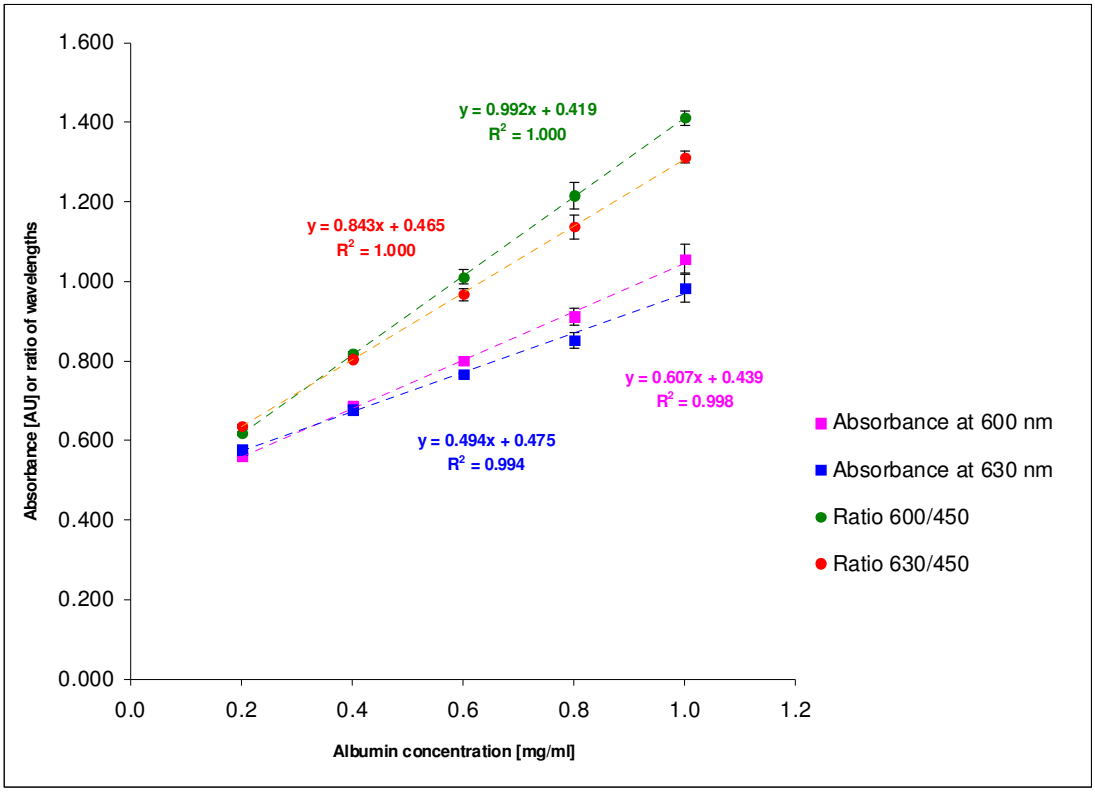


Figure 35 The calibration curve for albumin obtained on the plate reader by measuring the absorbance at 450, 600 and 630nm.

2.5.4.1. Enzyme concentration in the *E. coli* extract

The concentration of D-amino acid oxidase protein in the supernatant obtained from Ingenza, as determined by the Bradford assay (2.5.4), was 20mg·ml^{-1}. The concentration was calculated from the ratio of the two absorbances measured at

630nm and 450nm. The supernatant of DAAO was diluted 50-fold in four replications with water to fit the linear range of bovine serum albumin solutions used as a protein concentration reference (0.2-1.0mg·ml^{-1}).

2.5.4.2. Enzyme concentration in ATPS

Albumin in the concentration of 40, 70 and 100mg/ml was dissolved in each phase of 14/14% (w/w) PEG1000/ammonium phosphate and water as a control. Then proteins were analysed by the modified Bradford method (2.5.4). Figure 36 shows the ratio of OD_{630} to OD_{450} against albumin concentration in the three tested solvents. Values for water and the upper phase at all 3 concentrations are very close. The ratio obtained for the lower phase is much higher. Presumably, the high concentration of salt in the lower phase interferes with the Coomassie dye making the Bradford assay for the lower phase of the ATPS made of ammonium phosphate unsuitable.

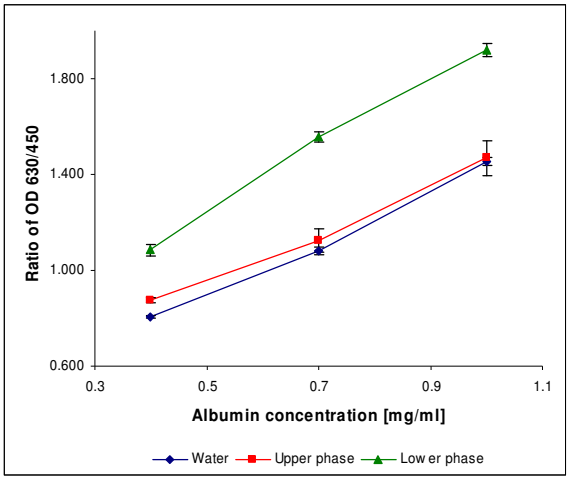


Figure 36 Comparison of Bradford protein assay in upper and lower phases of PEG/salt ATPS. The ratio of OD_{630} to OD_{450} of three solutions of albumin at concentrations: 40, 70 and 100mg/ml determined by the Bradford assay in water, upper and lower phases are plotted against concentration.

2.6. Determination of distribution ratio in a phase system

In this thesis, the distribution ratio (D) is expressed as the total concentration of an analyte in the upper phase $[A]_{upper}$ to its total concentration in the lower phase $[A]_{lower}$ at equilibrium.

$$D = \frac{[A]_{upper}}{[A]_{lower}} \qquad \textit{Equation 7}$$

However in terms of CCC the distribution ratio (*D*) is expressed as the total concentration of an analyte in the stationary phase to its total concentration in the mobile phase.

$$D = \frac{[A]_{stationary}}{[A]_{mobile}} \qquad \textit{Equation } 8$$

In the CCC-bioreactor the upper phase was used as the stationary phase, therefore:

$$D = \frac{[A]_{upper}}{[A]_{lower}} = \frac{[A]_{stationary}}{[A]_{mobile}} \qquad \textit{Equation } 9$$

Since either phase, can be used as the stationary phase, equation 9 is true only if the upper phase is used as the stationary one. Otherwise an inverse value needs to be used.

2.6.1. Distribution ratio of ABA and KBA in a phase system

To determine the distribution ratio, a solution of an analyte was added to a phase system. If the analyte was dissolved in water, the total volume added to the phase system was not higher than 0.5% of the phase system volume, to avoid altering the composition of the phase system markedly. The two phases were mixed and left for at least 10 min to achieve phase separation. Then the concentration of ABA/KBA was determined on HPLC. The distribution ratio was determined as follows:

$$D_{ABA} = \frac{[ABA]_{upper}}{[ABA]_{lower}} \qquad \textit{Equation } 10$$

$$D_{KBA} = \frac{[KBA]_{upper}}{[KBA]_{lower}} \qquad \textit{Equation } 11$$

2.6.2. Distribution ratio of the enzyme in a phase system

The distribution ratio of the enzyme was determined based on the enzymatic activity in both phases of a phase system. It was expressed as the enzyme activity in the upper phase to the activity in the lower phase at equilibrium following the colorimetric assay.

$$D_{enzyme} = \frac{Activity_{upperphase}}{Activity_{lowerphase}} \qquad \textit{Equation } 12$$

To determine the distribution ratio of the enzyme in a tested phase system the same amount (5ml) of both phases was placed into a glass vial. Next 50µL of the aqueous enzyme solution was added, shaken vigorously and left for about 10min to allow the two phases to separate. Then 10µL of the upper phase was placed into each of 3 wells of a 96 well plate. The same was done for the lower phase and then the enzyme activity was determined according to the procedure described in section 2.5.2.1.

As mentioned above, the D-value was determined based on the enzyme activity. Therefore it might happen that even if the enzyme was equally distributed between the phases, the determined distribution ratio based on the activity could be different from 1 if the upper or lower phase caused loss of enzyme activity to different degrees.

However all analysis were performed 10min after placing the enzyme into a phase system. This time was given to allow for separation of the two-phase system and was relatively short, after which the enzyme retained most of its initial activity, as illustrated in section 3.3.5.2. In addition to the distribution ratio, the enzyme stability in a tested phase system was determined. By repeated measurement over a given period of time, the exact value of D could only be determined for those systems in which the enzyme was stable. However, only for these systems was an exact D-value required, since only these could be used for the CCC-bioreactor. In the case of unstable systems, an approximate value could be generated, which was sufficient to observe some trends.

3. DEVELOPMENT AND OPTIMISATION OF A PHASE SYSTEM FOR THE CCC-BIOREACTOR

3.1. Introduction

Phase system selection plays a key role in the CCC separation as the technique is based on the differential partitioning between two immiscible phases. It is even more challenging to find an appropriate phase system for the CCC-bioreactor since there are other parameters that an ideal system must satisfy. The enzyme must totally partition into the stationary phase so it remains in the column, otherwise continual eluting with the mobile phase would cause the enzyme to contaminate the product. In addition, the solvent system should not cause enzyme activity degradation. The distribution ratio (D-value) for the substrate of the reaction (ABA) needs to be high so it stays it the column long enough to react. However, it cannot be too high so that its unreacted form (L-ABA) can elute from the column after a reasonable time. Finally the distribution ratio for KBA must be different from that of ABA so the two compounds elute at a different time.

There are some other important criteria for the solvent system selection that are related to the machinery of the CCC centrifuge. For example the viscosity of the phase system is one of the limiting factors as certain pumps, as well as narrow bore tubing (0.8mm), cannot cope with viscous solvents. Moreover the stationary phase must be retained in the column.

3.2. Overview of Chapter 3

In sections 3.3 to 3.5 there were three different groups of two-phase systems tested as a potential phase system for the CCC-bioreactor:

- Aqueous Two-Phase Systems (ATPS) as a solvent system for the CCC-bioreactor (section 3.3)
- Aqueous-organic systems as solvent systems for the CCC-bioreactor (section 3.4)
- Ionic liquids as solvent systems for the CCC-bioreactor (section 3.5)

Due to its mild nature and reported use in separations of biologically active compounds in CCC, ATPS based on polyethylene glycol (PEG) and a salt was a first choice as a phase system for the CCC-bioreactor.

Figure 37 shows the diversity of ATPS which can be obtained by using one of four molecular weight PEGs and one of seven salts, at various concentrations.

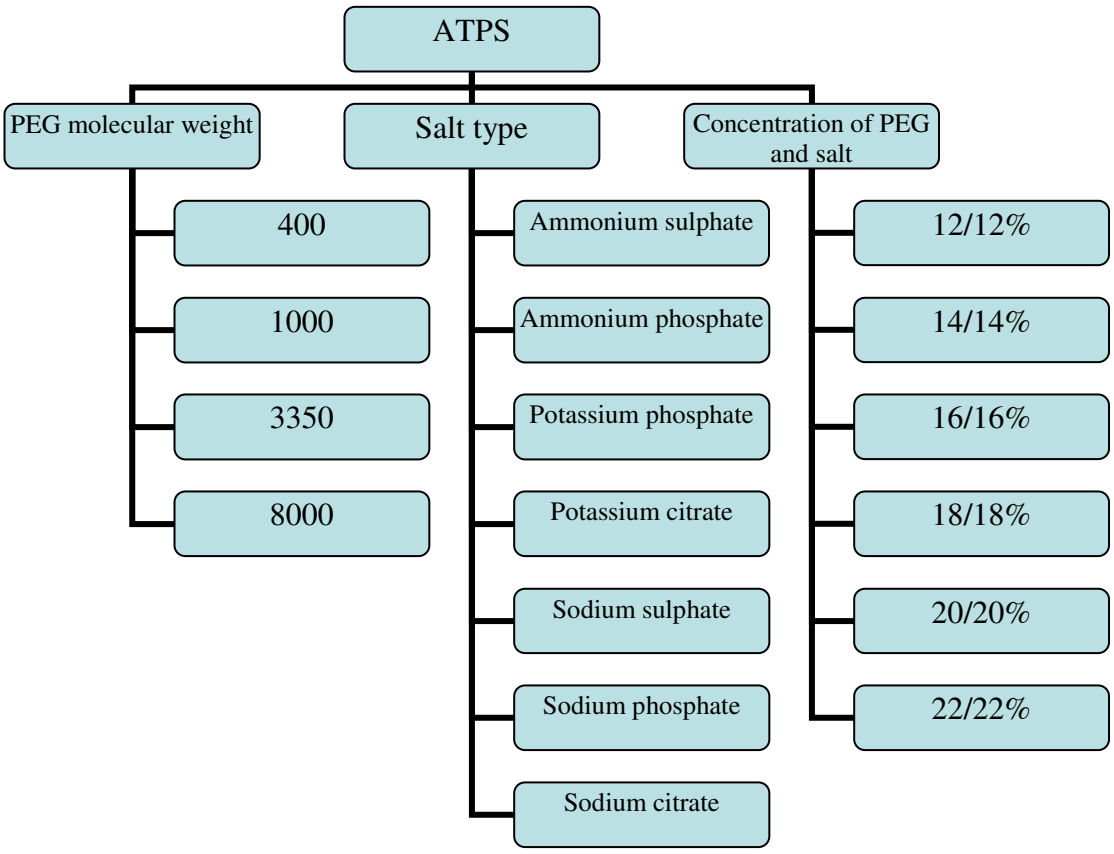


Figure 37 Parameters of ATPS made of PEG and salt that needed to be optimised to meet the criteria of a phase system for the CCC-bioreactor.

Based on the literature review and the project requirement, ammonium sulphate at a concentration of 18/18% w/w was chosen (3.3.1) as a phase system for the CCC-bioreactor. Unexpectedly, there was no bioconversion detected (3.3.1.4) when this phase system was applied. The enzyme was then found to be unstable in the selected phase system (3.3.1.6). Therefore, to find out more about the enzyme activity and stability in ATPS a further investigation involving a broad range of ATPS was performed (3.3.2 to 3.3.3).

After this research was completed, PEG was considered to be the factor destabilising the enzyme. Testing a broad range of ATPS in various concentrations, made of 3 different molecular weight PEG (3.3.3), gave rise to the indication of two potential phase systems for the CCC-bioreactor: 14/14% PEG3350/salt and 14/14% PEG1000/salt. The first phase system maintained the enzyme activity well but due to

unfavourable partitioning of the enzyme to the lower, salt-rich phase, it required the use of enzyme in the PEGylated form (3.3.4.1).

Having determined PEG1000 as the optimum polymer for the CCC-bioreactor, attention was then turned to the determination of the best salt. Ammonium sulphate, a salt component of ATPS used for the first CCC-bioreactor experiment, was replaced by potassium phosphate. The enzyme was found to be more stable in the presence of phosphate (3.3.5.2) and the potassium cation did not affect the chiral analysis of ABA as was found in the case of the presence of ammonium cations (2.5.1.2c).

ATPS 14/14% PEG1000/potassium phosphate, which due to the favourable partitioning of the enzyme did not require the use of its PEGylated form, was selected as a phase system for the CCC-bioreactor.

3.3. Aqueous Two-Phase Systems (ATPS) as a solvent system for the CCC-bioreactor

Because of its mild nature, the ATPS based on PEG1000 and a salt is the most commonly used solvent system for partitioning of biomolecules such as enzymes. In that kind of system, most proteins partition into the upper phase, which would suit the CCC-bioreactor. A PEG phase is usually easier to retain in the column, compared to a salt phase. Moreover the final product is easier to extract from a salt phase than from a PEG phase, e.g. using anion exchange columns.

3.3.1. ATPS of PEG1000/ammonium sulphate

Based on the literature review and other requirements, an aqueous two-phase system made of PEG1000 and ammonium sulphate was chosen as a phase system for the first testing of the CCC-bioreactor. The presence of ammonium ions was required for the next stage, which was a reduction of the KBA produced in the CCC-bioreactor to DL-ABA. This made a PEG1000/ ammonium sulphate system an obvious first choice.

Having decided which phase system to use for the preliminary studies, the salt and PEG concentration had to be selected. That was done based on ABA and KBA partitioning, as well as the ability of a system to be retained in CCC.

3.3.1.1. ABA partitioning in PEG1000/ammonium sulphate

The effect of ATPS concentration on the ABA distribution ratio was tested by determining the D-value of ABA in 5 different concentrations of ATPS made of PEG1000 and ammonium sulphate (Table 20). After settling of the two phases, samples were taken separately from the upper and the lower phase and analysed on HPLC for ABA according to the procedure described in the section 2.5.1.2b. There were no replications done as 5 concentrations were enough to get a trend line. Results are shown in Figure 38.

ATPS concentration [% w/w]	PEG1000 [g]	Ammonium sulphate [g]	Water [g]	0.2M ABA [ml]
14/14	1.4	1.4	6.7	0.5
16/16	1.6	1.6	6.3	0.5
18/18	1.8	1.8	5.9	0.5
20/20	2.0	2.0	5.5	0.5
22/22	2.2	2.2	5.1	0.5

Table 20 Composition of ATPS used to determine ABA distribution ratio.

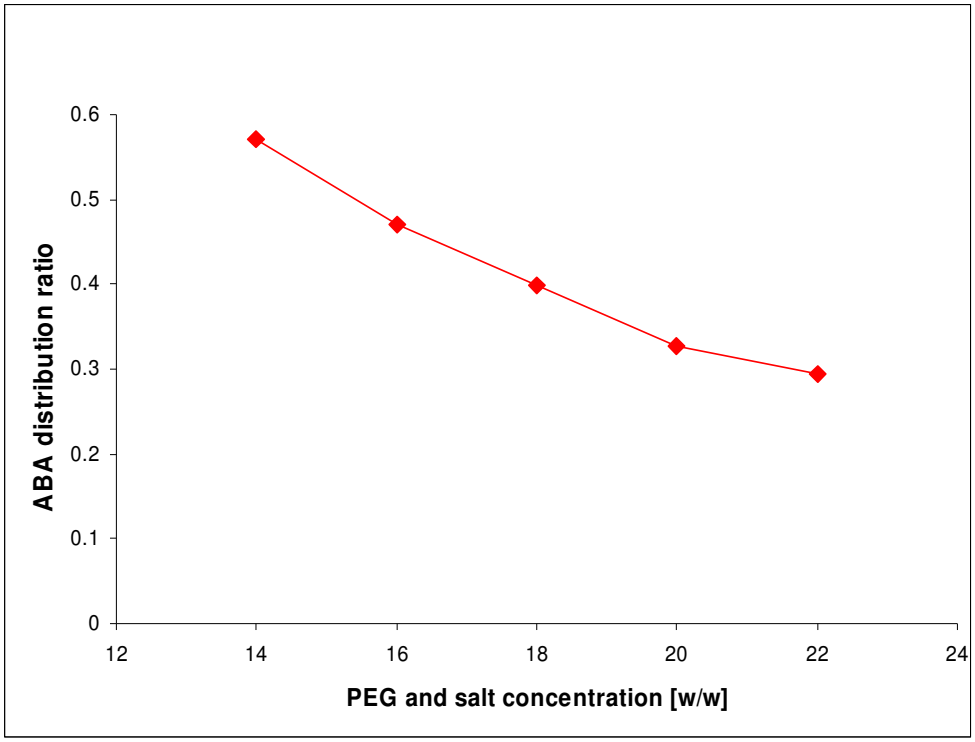


Figure 38. Partitioning of aminobutyric acid (ABA) in different concentration APTS made of PEG1000/ammonium sulphate. The distribution ratio was determined by dividing the peak area of ABA in the upper phase by the ABA peak area in the lower phase.

The higher the PEG and the salt concentration in the ATPS, the less ABA partitioned into the top phase. For the CCC-bioreactor the D-value of ABA should be high so it stays in the stationary phase long enough to react. For this reason a lower concentration ATPS, where more ABA partitions to the PEG phase, would be more suitable.

3.3.1.2. KBA partitioning in PEG1000/ammonium sulphate

The effect of ATPS concentration on KBA partitioning was examined. That was done for 6 concentrations of ATPS made of PEG1000 and ammonium sulphate.

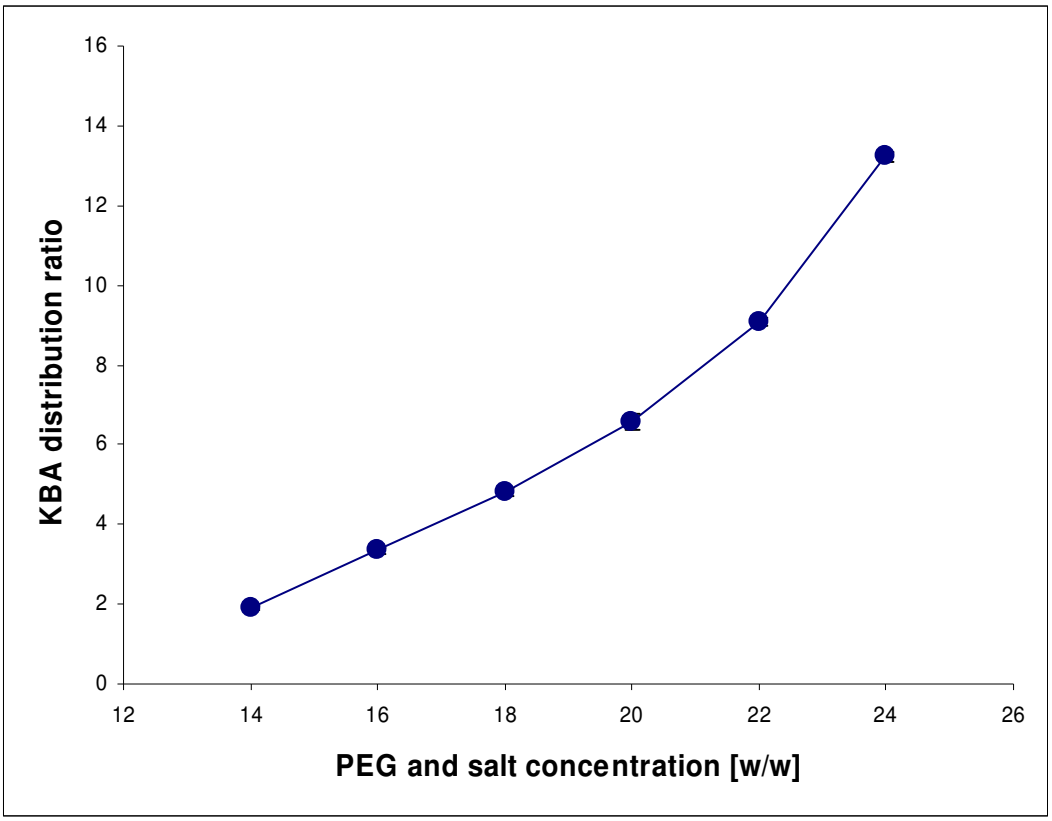


Figure 39 Partitioning of ketobutyric acid (KBA) in different concentration APTS made of PEG1000/ ammonium sulphate. The composition of the phase system is presented in Table 20, ABA solution was replaced by 0.2M KBA solution. The upper and the lower phase were analysed on HPLC for KBA using an anion exchange column (2.5.1.3). The distribution ratio was obtained by dividing the peak area for KBA in the upper phase by the KBA peak area in the lower phase.

Most KBA partitioned into the top, PEG-rich phase. The higher the PEG and salt concentration, the more KBA molecules goes to the top phase (Figure 39). The higher the D-value, the more time is required to elute KBA from the CCC.

3.3.1.3. Retention of the phase in CCC using PEG1000/ammonium sulphate

To obtain a good resolution in CCC, a reasonable percentage of the stationary phase must retain in the CCC coil. Even if there is no specification regarding the amount of the stationary phase in the coil, it is known that the more stationary phase in the coil, the better the resolution[148].

Using aqueous organic two-phase systems, Wood[149] demonstrated a linear correlation between the volume of the stationary phase displaced and the square root of the mobile phase flow rate.

To determine the retention of the ATPS made of PEG1000 and ammonium sulphate on the "Milli" CCC machine, retention studies were performed for 4 different concentrations of ATPS: 16/16, 18/18, 20/20 and 22/22% w/w. The top PEG-rich phase was used as the stationary phase. Initially the coil was entirely filled with that phase. The coil was then rotated in clockwise direction at 1800 rpm. The mobile phase was pumped (head to tail) through the rotating coil, partially displacing a portion of the PEG-stationary phase. The volume of the displaced stationary phase was recorded after equilibrium was achieved, when there was no more of the stationary phase coming out from the coil. The same was done for higher flow rates. The volume of the stationary phase retained in the coil (stationary phase retention) was plotted against the square root of a flow rate to give a linear correlation[149]. Results are presented in the Figure 40.

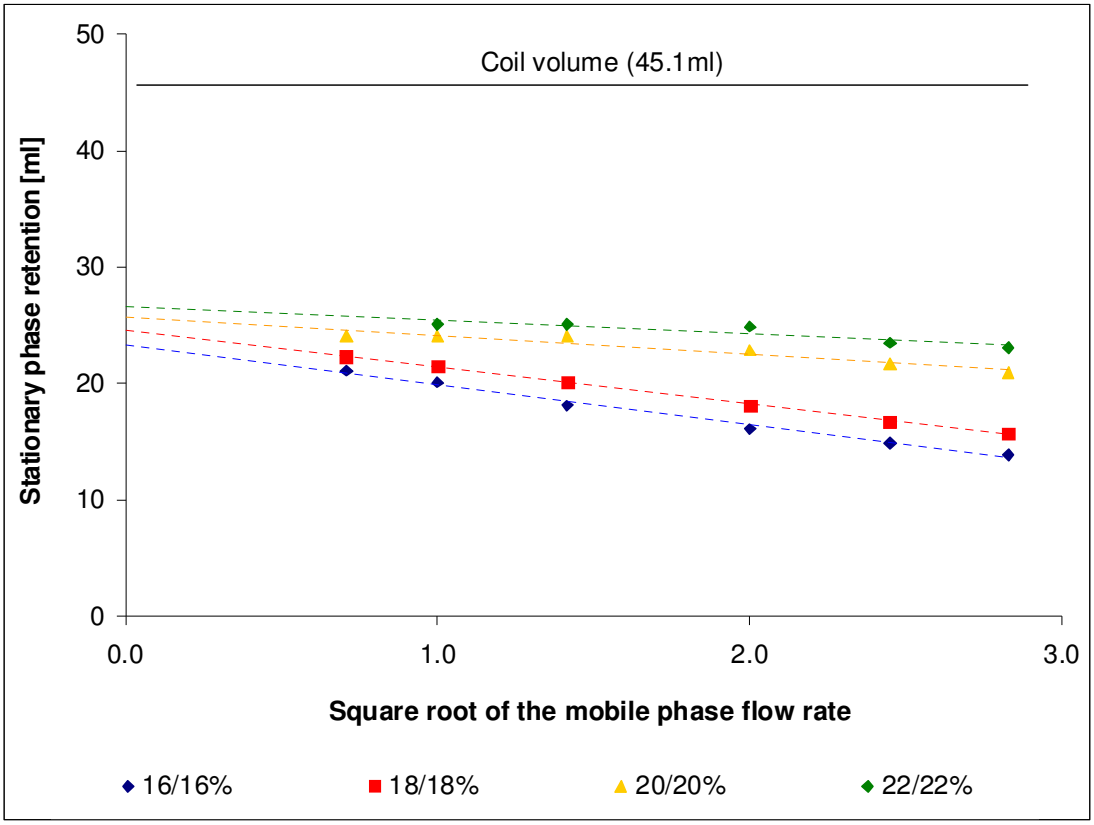


Figure 40 Retention studies of four concentrations of ATPS made of PEG1000 and ammonium sulphate tested in "Milli" CCC, 45.1ml coil volume, 2.7mm bore. The mobile phase was pumped in the Head to Tail direction. Initially the coil was entirely filled with the upper phase. Then the mobile phase was pumped at a very low flow rate through the rotating 1800rpm coil. The displaced stationary phase was collected in a measuring cylinder till equilibrium was reached, when no further stationary phase came out. The volume of the displaced stationary phase was recorded. Then the flow rate of the mobile phase was increased and the mobile phase pumped till equilibrium for that flow rate was reached and the additional volume of the stationary phase displaced at that flow rate noted.

It was possible to retain ATPS 14/14% PEG1000/ammonium phosphate on the CCC coil. Moreover the stationary phase retention was stable and depended on both the ATPS concentration and the mobile phase flow rate. The Wood plot performed for this phase system shows that the volume of the stationary phase displaced depends linearly on the square root of the mobile phase flow rate. The higher the ATPS concentration, the better the stationary phase retention. This results from a larger density difference between the two phases in more concentrated ATPS. Du *et al*[150] have shown that for organic solvents, the stationary phase retention decreases proportionally to the square root of the mobile phase flow rate. That was also true for the ATPS PEG1000/ammonium sulphate (Figure 40). As expected the higher the

mobile phase flow rate, the more stationary phase was displaced, thus the lower the stationary phase retention.

Using the data obtained from the experiments above, the theoretical retention time of ABA and KBA for different concentrations of ATPS and different mobile phase flow rates was calculated (Table 21). The retention time is proportional to the D-value and inversely proportional to the flow rate. Therefore in the case of KBA, whose D-value strongly depends on ATPS concentration (Figure 39), the higher the ATPS concentration, the greater the KBA elution time. There is an opposite trend for ABA, which would elute earlier if a higher ATPS concentration was applied. Since the D-value slightly decreases as the ATPS concentration goes up (Figure 38), changing ATPS concentration slightly affects the ABA elution time (Table 21). Therefore selection of ATPS concentration was made mainly based on KBA retention time. The standard mobile phase flow rate for a 45.1ml CCC coil is 1ml/min. At that flow rate the KBA would elute after 139min if 18/18% ATPS was used. In the case of 20/20%, 197min was regarded as an excessively long run time. If 16/16% ATPS was used, KBA would elute after 99min, which is acceptable, however for this concentration, the stationary phase retention is the poorest (Figure 40), which will affect the resolution[148]. Therefore, the phase system 18/18% w/w PEG1000/ammonium sulphate was selected as a compromise.

Flow rate [ml/mim]	Theoretical retention time [min]							
	ABA				KBA			
	ATPS concentration [%w/w]				ATPS concentration [%w/w]			
	16/16	18/18	20/20	22/22	16/16	18/18	20/20	22/22
0.25	135				416			
0.5	69	64	58		203	284	394	
1	35	32	29	27	99	139	197	272
2	18	17	15	14	47	67	99	136
4	9	9	7	7	23	32	48	68
6	6	6	5	5	15	20	31	43
8	5	4	4	4	11	15	22	32
10			3	3			18	25

Table 21 The theoretical retention time of ABA and KBA in CCC based on the stationary phase retention, distribution ratio and a flow rate calculated according to Equation 3 (section 1.3.3). The volumes of the mobile phase (V_M) the stationary phase (V_S) were calculated based on the stationary phase displaced (Figure 40) using *Equation* 4 and *Equation* 5. The phase system used was ATPS PEG1000/ammonium sulphate. The upper PEG-rich phase was used as the stationary. The mobile, lower, salt-rich phase was pumped from head to tail.

3.3.1.4. CCC-bioreactor using PEG1000/ammonium sulphate

Having selected the solvent system, the next stage was performing the CCC-bioreactor. Since the subsequently developed chiral method of ABA analysis was not available at that time, only one enantiomer, D-ABA was used to perform the first CCC-bioreactor.

The substrate (D-ABA) was injected onto the equilibrated CCC column, with the enzyme, DAAO, in the stationary, PEG-rich phase, to convert D-ABA into KBA. The mobile phase coming out from the CCC was passed through a UV-detector and the signal (λ=220nm) was recorded. In addition the mobile phase was analysed off line for KBA using HPLC.

In the chromatogram from the UV-detector, (Figure 41), there is a single peak for ABA, which eluted after 31min. The theoretical elution time for ABA, based on its distribution ratio, the displaced stationary phase volume and the mobile phase flow rate, was 32min. There is no peak for KBA, which was expected to elute at 139min. Furthermore HPLC analysis (2.5.1.3) did not indicate any presence of KBA in any of analysed fractions. Thus no bioconversion took place.

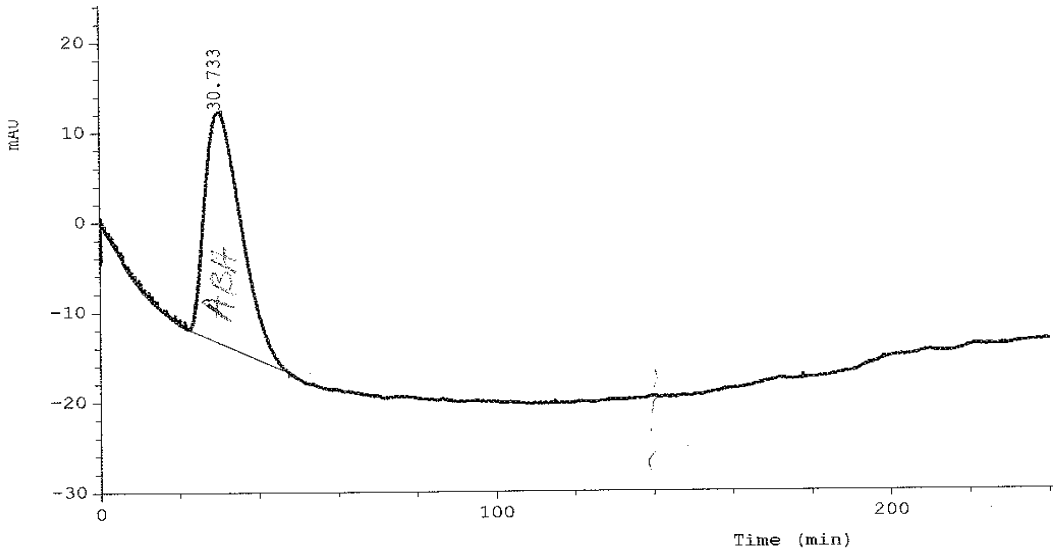


Figure 41 Chromatogram obtained from UV detector at 220nm on the CCC-bioreactor. 47.1mg D-ABA was injected onto the column. Temperature 30°C, rotational speed 1800rpm. Stationary phase displaced 23ml (S_f =51%). The stationary phase was the top phase with the enzyme, DAAO (2ml of the enzyme supernatant dissolved in 98ml of the upper phase). The mobile phase was the lower phase of 18/18% PEG1000/ ammonium sulphate (pH 7.6) pumped from head to tail direction at a flow rate 1ml/min.

3.3.1.5. Enzyme activity in PEG1000/ammonium sulphate

In was presumed that the failure of CCC-bioreactor, presented in the previous section, might have been caused by a low enzyme activity in the selected phase system. Therefore the conversion of ABA in 18/18% PEG1000/ammonium sulphate was performed in a test tube, rather then in the CCC-bioreactor. The KBA produced by enzyme activity was analysed on HPLC.

The enzyme activity was analysed separately for the upper and the lower phase. Moreover, to investigate the effect of the phase system pH on the activity, the experiment was performed at the pH of the phase system (pH 5) and also at pH 7.5, the optimum pH for DAAO[151], achieved by adding a drop of ammonia solution.

Into each test tube was placed 4.8ml of a phase (top or bottom) or water (control), 100µL of the enzyme supernatant and 100µL of a substrate solution containing 10mg D-ABA. The enzyme activity was determined by HPLC by analysing the KBA produced as a result.

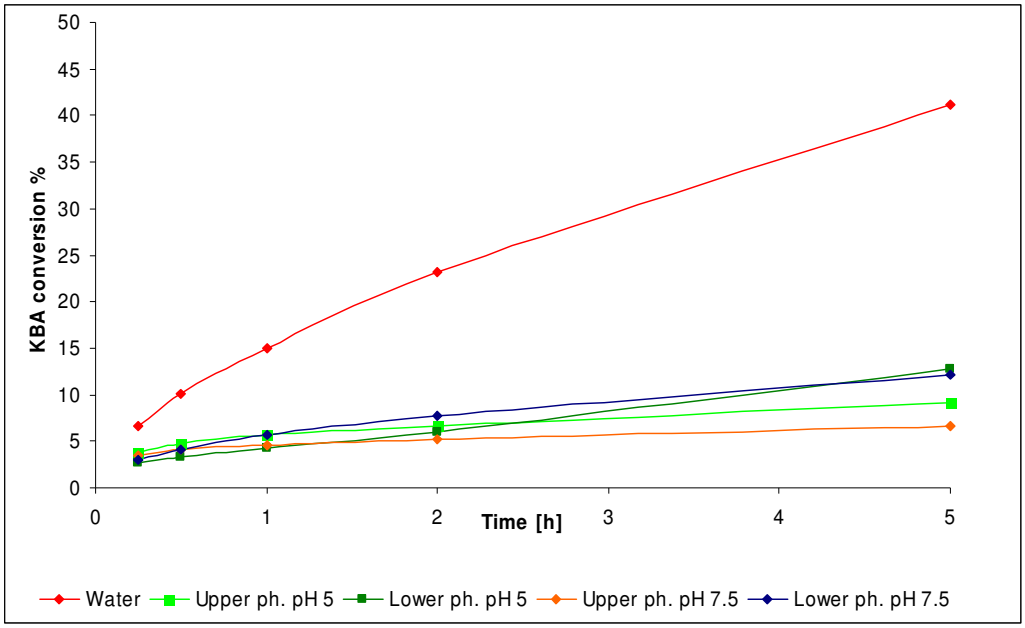


Figure 42 DAAO activity in the ATPS 18/18% w/w PEG1000/ammonium sulphate determined by KBA produced by enzymatic activity as analysed on HPLC (anion exchange column)

Figure 42 shows the DAAO activity in the ATPS 18/18% w/w PEG1000/ ammonium sulphate at the two tested pH. The enzyme activity in the ATPS was much lower than in the control (water) at both pH. Thus the pH of a phase does not seem to

be a key parameter in the enzyme activity in ATPS. A very low enzyme activity in the tested phase system can explain the lack of KBA in the mobile phase coming out from the CCC-bioreactor (Figure 41), especially as the substrate (ABA) elutes after only 32min.

3.3.1.6. Enzyme stability in PEG1000/ammonium sulphate

Since it was discovered that the enzyme was not very active in the ATPS 18/18% PEG1000/ammonium sulphate (3.3.1.5), the enzyme stability in that system was determined. ATPS was initially chosen as a solvent system because of its mild nature. No publications had been found indicating that enzyme instability might be caused by an ATPS. Therefore it was suspected that the enzyme instability might have been caused by some contaminant present in the PEG. For that reason enzyme stability was tested in ATPS made of three different PEG1000, purchased from two different suppliers, Merck and Fisher. PEG purchased from Merck was in two different grades; for synthesis (F. synth.) and Ph Eur.

The stability was determined by measuring the enzyme activity after exposure to the upper phase, lower phase and 50% aqueous solution, for 0, 1 and 2 hours. The concentration of PEG in aqueous solution was chosen to be 50% as that was the maximum estimated concentration of PEG in the upper phase. The enzyme supernatant was placed separately into the tested solutions using a colorimetric assay (2.5.2.1). For a control, the activity of the enzyme solution in water was tested.

	Time	Enzyme activity (absorbance/min)									
		Water	Merck Ph Eur UP	Merck Ph Eur LP	Merk Ph Eur PEG50%	Merck F.synth. UP	Merck F.synth LP	Merk F.synth PEG50%	Fisher UP	Fisher LP	Fisher PEG 50%
Activity [abs/min]	0	0.153	0.130	0.146	0.169	0.116	0.136	0.160	0.127	0.134	0.150
	1	0.141	0.009	0.065	0.146	0.007	0.069	0.118	0.005	0.082	0.142
	2	0.138	0.003	0.040	0.141	0.002	0.040	0.119	0.002	0.049	0.141
St. error	0	0.002	0.004	0.001	0.003	0.003	0.003	0.002	0.002	0.003	0.005
	1	0.002	0.000	0.002	0.001	0.000	0.001	0.002	0.000	0.001	0.005
	2	0.002	0.000	0.000	0.001	0.000	0.002	0.004	0.000	0.001	0.001

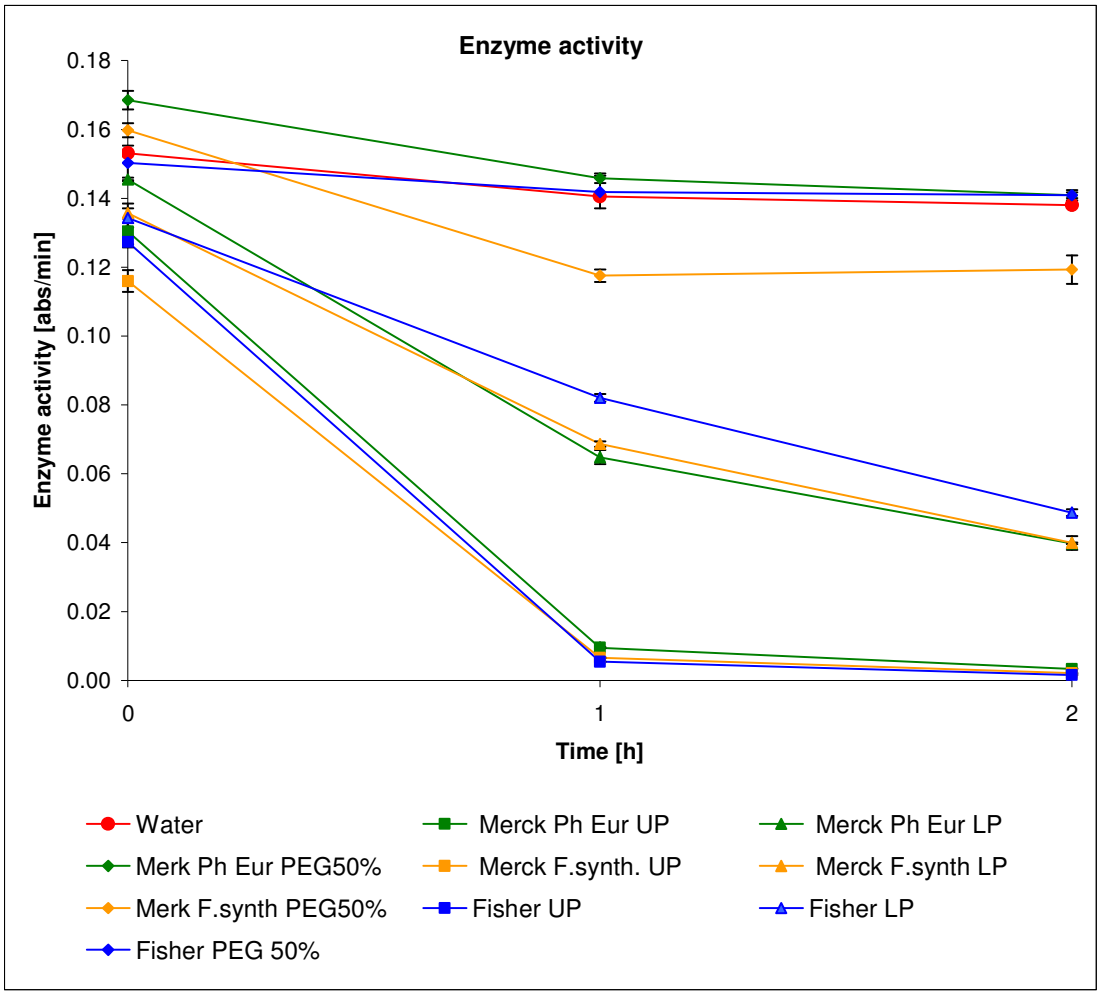


Figure 43 DAAO stability in ATPS made of 18% ammonium sulphate and 18% PEG1000 from different sources. The enzyme supernatant (50µL) was dissolved in 4950µL of tested phase. The enzyme activity was measured by the colorimetric assay after 0, 1 and 2 hours exposure to the tested phase.

The enzyme was found to be unstable in both phases of the ATPS 18/18% PEG1000/ammonium sulphate (Figure 43). The PEG supplier, as well as purity grade, did seem to make any difference. The biggest inactivation was noticed in the upper phase, where within 1 hour the enzyme lost 94% of the initial activity. That is unfavourable for the CCC-bioreactor, where the enzyme is distributed in the upper phase. That might explain the failure of the CCC-bioreactor described in section

3.3.1.4, as by the time the substrate was injected onto the CCC column, the enzyme had been in the stationary phase for about 40min and was presumably already inactivated.

Also the lower phase caused loss of enzyme activity. After 1h there was half of the initial activity (48% loss). Interestingly, the enzyme is quite stable in 50% PEG solutions (15% loss). For comparison, in the same time the enzyme dissolved in water lost only 8% of its initial activity. Since the upper phase made of PEG1000 and ammonium sulphate inactivates the enzyme the most, further investigation focussed on other phase systems, where the enzyme may remain active.

3.3.2. Enzyme stability in ATPS

Having identified the enzyme instability in ATPS as a potential drawback of the CCC-bioreactor, further investigations were done using a broad range of different ATPS. Therefore the next studies were done to understand which factors cause enzyme inactivation.

3.3.2.1. Enzyme stability in PEG solutions

From Figure 43 it was concluded that the enzyme is particularly unstable in the upper phase. Given that the upper phase is a PEG-rich phase, the enzyme stability was determined in various solutions of different molecular weight PEG (polyethylene glycol):

- PEG400 5% (pH 7.7), 15% (pH 6.4) and 30% (pH 7.2)
- PEG1000 5% (pH 7.2), and 30% (pH 7.4)
- PEG8000 5% (pH 7.1), 15% (pH 7.0) and 30% (pH 7.5)

The studies revealed that the enzyme stability might be dependent on the PEG molecular weight (Figure 44). The enzyme stayed totally active in a solution of 30% PEG8000 but was losing activity in 30% solutions of PEG1000 and PEG400 (Figure 44d). After 2 hours incubation of the 30% PEG solutions, the activity expressed as % of the initial activity was 69% in PEG400, 81% in PEG1000 and 109% in PEG8000. For PEG400 and PEG1000, concentration of the solution also affected the enzyme activity (Figure 44a and b). The inactivation was quicker in a more concentrated PEG400 solution. The enzyme seems to be stable in 5% and 15% PEG400 solution but lost its activity in 30% solution (Figure 44a). The highest activity was retained in

the presence of PEG8000. It is worth noting that the enzyme seems to be more stable in aqueous solutions of PEG8000 than in water (Figure 44c).

The experiment showed that the enzyme might not be stable in aqueous solutions of low molecular weight PEG, especially PEG400. Based on these results, PEG8000 appears the best to use as a component of ATPS for the CCC-bioreactor, with PEG1000 second.

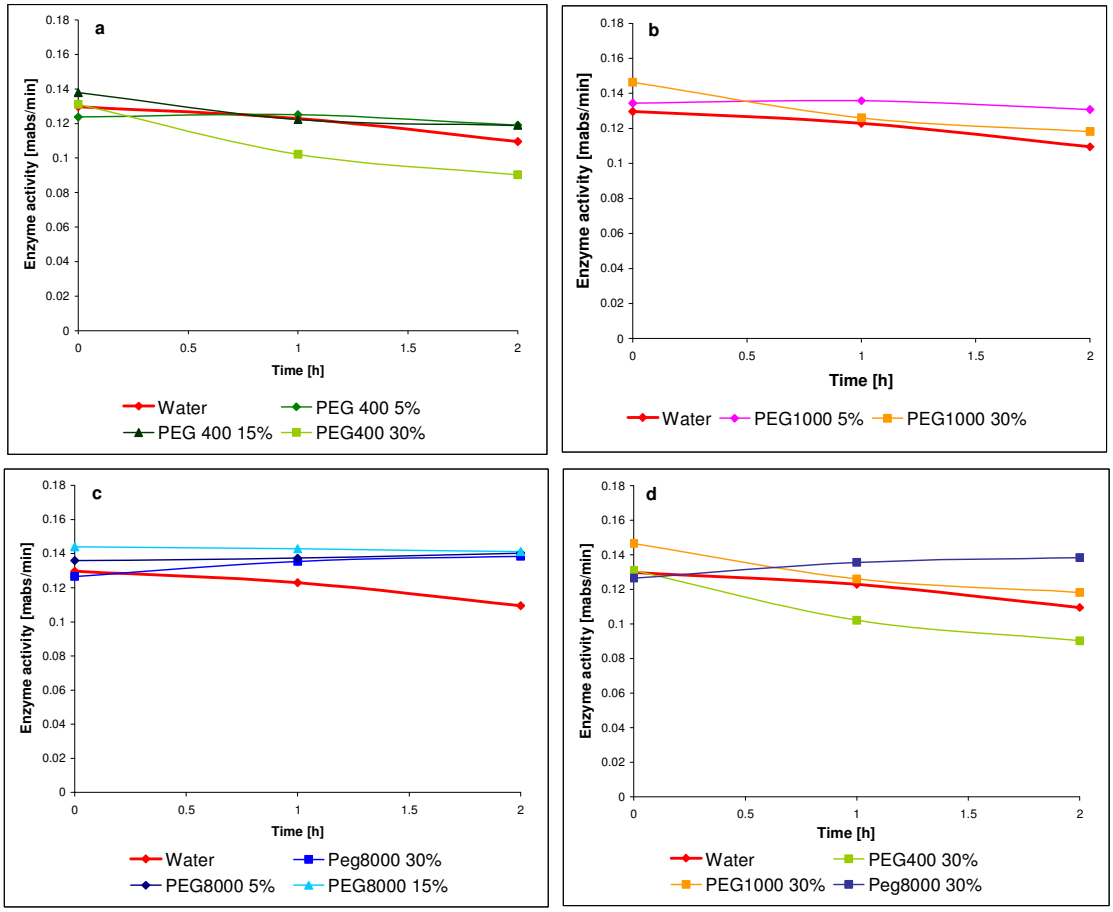


Figure 44 PEG molecular weight effect on the enzyme stability; a) Effect of PEG400 concentration on the enzyme stability, b) Effect of PEG1000 concentration on the enzyme stability, c) Effect of PEG8000 concentration on the enzyme stability, d) PEG molecular weight effect on the enzyme stability determined in 30% PEG solutions (PEG 400, PEG 1000, PEG 8000)
50µl of the enzyme as dissolved in 4950µl of a PEG solution. The enzyme activity was determined by the colorimetric assay (2.5.2.1) at the time 0, 1 and 2h.

3.3.2.2. Enzyme stability in salt solutions

The enzyme stability was also determined for 40% ammonium sulphate solution and 40% ammonium phosphate solution. It is known that the concentration of salt in the salt phase of the ATPS 18/18% PEG1000/ammonium sulphate is of the order of 40%. The enzyme was found to be stable in both tested solutions of ammonium sulphate and ammonium phosphate (Figure 45). The activity measured after 2h was equal to the initial activity.

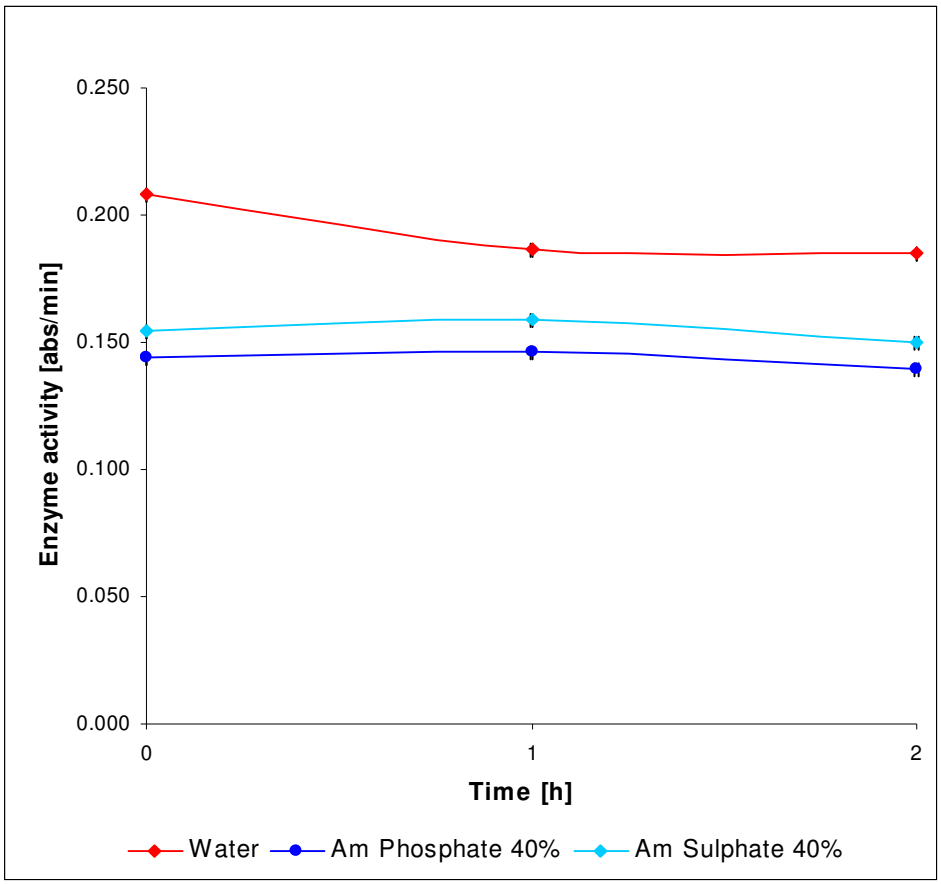


Figure 45 DAAO stability in 40% ammonium sulphate and 40% ammonium phosphate solutions. 50µl of the enzyme as dissolved in 4950µl of a salt solution. The enzyme activity was determined by the colorimetric assay (2.5.2.1) at the time 0, 1 and 2h.

3.3.3. Relationship between PEG molecular weight/concentration, enzyme stability and the partitioning of all components

At this stage a successful CCC-bioreactor run had still not been achieved. This was considered to be due to the loss of activity of the enzyme in the PEG phase. It was previously demonstrated (3.3.2.1) that the enzyme is totally stable in aqueous

solutions of PEG8000. However, it was losing activity in solutions of PEG400 and PEG1000, especially at higher concentrations (30%) as found in the upper phase of ATPS (Figure 44). For that reason it was reasonable to test the enzyme activity and the distribution ratio in ATPS made of PEG with molecular weights higher than 1000. Since solutions of PEG8000 are very viscous and therefore difficult to use in CCC, PEG3350 was tested as a compromise molecular weight, compared in each case to the PEG8000 in which the enzyme was known to be stable.

It seemed that there were two factors that affected the enzyme stability, PEG molecular weight and PEG concentration (3.3.2.1). Therefore, an extensive analysis of the enzyme stability was performed in a range of ATPSs made of different molecular weight PEG (400, 1000 and 3350) and potassium phosphate salt at the concentration of 12/12, 14/14, 16/16 and 18/18 % w/w. This salt was used as it was compatible with the chiral derivatisation of ABA (2.5.1.2c). PEG400 at the concentration of 12/12% and 14/14 % did not made a two-phase system with potassium phosphate. Since other important parameters in phase system selection are the distribution ratios of the enzyme, ABA and KBA, these were also determined. All the experiments were done in triplicate and results are presented in the Table 22.

PEG mol. weight	ATPS * concentration [% w/w]	Enzyme stability **	SD	Distribution ratio					
				Enzyme		ABA		KBA	
				D-Value	SD	D-Value	SD	D-Value	SD
PEG 400	16/16	27.3	6.0	15.7	2.2	0.9	0.04	2.2	0.19
	18/18	13.0	2.4	15.9	3.8	1.2	0.12	4.8	0.29
PEG 1000	14/14	30.4	1.9	15.2	3.0	0.6	0.01	2.2	0.03
	16/16	7.8	0.5	4.5	1.3	0.6	0.05	3.4	0.10
PEG 3350	12/12	87.2	1.5	0.1	0.0	0.6	0.03	2.0	0.02
	14/14	87.4	3.5	1.5	0.1	0.4	0.03	2.7	0.03
	16/16	75.2	4.3	21.2	9.2	0.3	0.02	3.7	0.05

Table 22 Effect of PEG molecular weight and ATPS concentration for ATPS of PEG and potassium phosphate on the stability of DAAO and the distribution ratios of the enzyme, ABA and KBA.

*ATPS concentration expressed as % w/w PEG/potassium phosphate pH 7.6

**enzyme stability is a % of the initial activity incubation of enzyme for 2 hours in a phase

The conclusions were as follow:

- The enzyme is more stable in ATPS made of PEG 3350 and less stable in ATPS made of PEG400
- ATPS concentration plays a key role in the enzyme stability. Enzyme is more stable in less concentrated ATPS (true for all molecular weight PEG)
- Distribution ratio of KBA increases with PEG molecular and with increasing ATPS concentration
- Distribution ratio of ABA decreases with ATPS concentration

Regarding the ability to maintain the enzyme activity, the best ATPSs are made of PEG3350. A problem that would arise from using those phase systems in the CCC-bioreactor comes from unfavourable partitioning of the enzyme to the lower, salt-rich phase. Although, at the concentration of 16/16% w/w the D-value of the enzyme is 21, there was a big inconsistency between both the three replications (SD=6.2), as well as between 3 wells on a microplate into which a sample from the same replication was placed. Furthermore, the upper phase was very cloudy and phases seemed not to be separated properly. In addition, partitioning of ABA and KBA were not as favourable as in other phase systems. ABA with the D-value of 0.3 might not stay long enough in the coil to be totally converted before it elutes. In turn, the D-value of KBA 3.7 means a long retention time which would delay a potential second injection of substrate.

Both ABA and KBA partition more favourably in ATPS 14/14% PEG3350/salt. Also the enzyme seems to be more stable in this phase system, however it does not partition into the upper, PEG-rich phase. Using this phase system would require a method such as PEGylation of the enzyme, which would drive it to the top, PEG-rich phase.

Other potential phase systems that arise from the data in Table 22 are ATPS made of PEG400 at the concentration of 16/16% and PEG1000 at the concentration of 14/14%. Distribution ratios of ABA and KBA are more favourable in these phase systems than in PEG3350/salt at the concentration of 16/16% or even 14/14%. The D-value of ABA is higher in comparison to the previously mentioned phase systems, which means a longer retention time and as a consequence of this, a longer reaction time. In turn, the D-value of KBA is lower than that obtained for 16/16% or 14/14%

PEG3350/salt, which is preferred as the compound would require less time to elute. This would reduce both the time of the separation process and the mobile phase consumption. The drawback of these phase systems is their poor ability to maintain the enzyme activity compared to PEG3350/salt. ATPS made of PEG1000 can be used a concentration as low as 14/14%, which reduces PEG and salt consumption, compared to PEG400 which needs to be used at a concentration of 16/16% (w/w). Therefore, ATPS made of PEG1000 rather then PEG400 was further investigated.

In the next sections the ability of the two selected two-phase systems (14/14% PEG3350/salt and 14/14% PEG1000/salt) for the CCC-bioreactor will be tested.

3.3.4. ATPS of PEG3350 with enzyme PEGylation

One of the two phase systems selected for the CCC-bioreactor was 14/14% PEG3350/salt (3.3.3). Due to unfavourable partitioning of the enzyme to the lower, salt-rich phase, a method which alters the partition of the enzyme had to be developed and proven in the selected phase system. This was PEGylation.

In the next sections will be described the methodology of enzyme PEGylation (3.3.4.1), the distribution ratio of the PEGylated enzyme in ATPS made of PEG3350 (3.3.4.2) and the activity (3.3.4.3) and stability (3.3.4.4) of the PEGylated enzyme. Finally the effect of salt type on PEGylated enzyme will be described (3.3.4.5).

3.3.4.1. Methodology of enzyme PEGylation

PEGylation is the process of covalent attachment of polyethylene glycol to another molecule. It is commonly used to mask the surface of therapeutic proteins to avoid their removal by exposure to antibodies and reduction of renal clearance[152].

Gavasane and Gaikar[153] (2003) examined several different PEG-derivatives (PEG-benzonate, PEG-phosphate, PEG-trimethylamine, PEG-palmitate, PEG-phenylacetamide) to extract penicillin acylase from *E. coli* using ATPS made of 10% PEG4000 and 7.5% sodium sulphate. Penicillin acetate, in the tested ATPS, partitioned into the bottom salt phase. Adding any of the PEG-derivatives above a certain concentration drove the target protein to the upper-PEG phase. The same phase system was used to purify glucose isomerase which partitioned into the top-PEG phase in the presence of PEG-benzoate and PEG-palmitate[154].

Slavica *et al*[155] (2007) performed PEGylation of DAAO from *Trigonopsis variabilis* using polyethylene glycol maleimide (MPEG-Mal), whose structure is presented in Figure 46. The PEGylation was performed in the presence of 50mM sodium phosphate buffer, pH 7.5, using a two-fold molar excess of reactive PEG over protein cysteine groups. The reaction was completed immediately after adding MPEG-Mal to the enzyme. In SDS-PAGE the PEGylated enzyme migrated as a single band whose molecular mass increased by ≈15 kDa compared with the native enzyme (55 kDa PEGylated protein, 40 kDa native enzyme). This suggested that each molecule of the enzyme was combined with three molecules of MPEG-Mal.

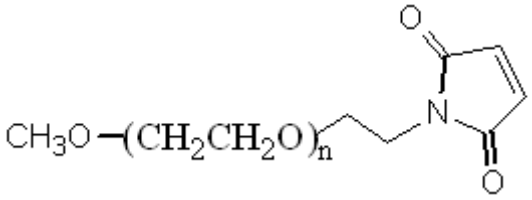


Figure 46 The structure of PEG maleimide (MPEG-Mal), n≈110.

In this project, the PEGylation of the DAAO enzyme was achieved as follows: 200µL of DAAO obtained from Ingenza (0.1µM subunits) was incubated for 10 min at 4°C with 6 mg of MPEG-Mal (1.2µM subunits) in the presence of 0.8ml of 50mM sodium phosphate buffer, pH 7.5. Proportions of DAAO to MPEG-Mal were the same as used by Slavica and co-workers[155].

3.3.4.2. Distribution ratio of PEGylated enzyme in PEG3350/salt

The effect of MPEG-Mal on the enzyme distribution ratio in the ATPS (12/12% PEG3350/ammomium phosphate) was tested. ATPS at the concentration of 12/12% was used to demonstrate the contrast in partitioning of the native enzyme, which in this phase system partitions almost entirely into the lower phase (Table 22) and the PEGylated form. At this stage of the research, ammonium phosphate was still used as the salt component of ATPS.

50µL of the PEGylated enzyme was mixed with 475µL upper phase and 475µL lower phase of the ATPS (12/12% PEG3350/ammonium phosphate). After vigorously shaking and settling the two phases, the enzyme activity was determined for both

phases in the colorimetric assay (2.5.2.1). As a control, a solution of non-PEGylated enzyme was placed in the ATPS.

Results (Figure 47) showed, as expected, that when non-PEGylated enzyme was used there was much more activity in the lower phase (0.253abs/min, 94.8%) than in the upper phase (0.014abs/min, 5.2%). At the same time there was a high enzyme activity (0.186abs/min, 93%) in the upper phase when the PEGylated enzyme was placed into the ATPS. The activity in the lower phase was 0.014abs/min, 7%. This suggested that PEGylation drives the enzyme from the lower salt-rich phase to the upper-PEG-rich phase. Based on the activity, the distribution ratio of the enzyme was calculated. The distribution ratio of the PEGylated enzyme was found to be 13, an increase of over 200-fold compared to the control.

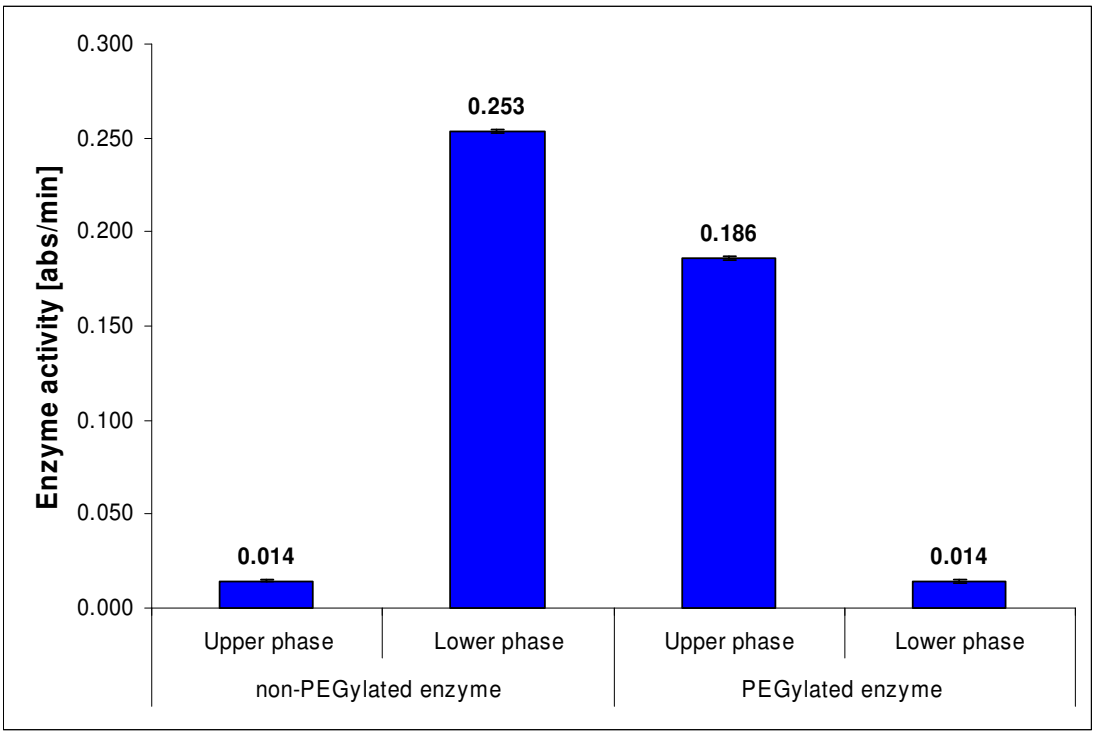


Figure 47 The effect of DAAO PEGylation with MPEG-Mal on the partitioning in the ATPS (12/12% w/w PEG3350/ammonium phosphate) determined by the colorimetric assay.

3.3.4.3. Activity of PEGylated enzyme

Using the chiral HPLC analysis of D/L-ABA, the activity of PEGylated DAAO in the ATPS and a buffer was compared to the activity of the native (non-PEGylated) form of DAAO. These made four combinations which were tested in duplicate.

In all 4 cases a reaction volume was 2030µl. 1920µl of this was the ATPS (960µl of each phase) or the buffer. 10µl was DL-ABA solution (130.9mg/ml). PEGylated enzyme (3.3.4.1) was added in a volume of 100µl, which contained 20µl of the enzyme. The native enzyme was used in the volume of 20µl together with 80µl of water added to obtain the same final volume of 2030µl in all vials. The reaction was carried out for 10min at room temperature and stopped by the chiral derivatisation (2.5.1.2d) of 100µl of a sample. Results can be found in Table 23.

			Peak Area [mVs]		D-ABA converted [%]	Mean conversion [%]
			L-ABA	D-ABA		
Native	Dried enzyme	Rep. 1	1261	703	44	45
		Rep. 2	1169	641	45	
	Control	Rep. 1	1264	566	55	56
		Rep. 2	1233	536	57	
PEGylated	Dried enzyme	Rep. 1	1233	1115	10	10
		Rep. 2	1192	1072	10	
	Control	Rep. 1	1217	1072	12	12
		Rep. 2	1193	1047	12	

Table 23 Activity of the PEGylated DAAO and the native form of DAAO in a buffer (50mM sodium phosphate, pH 7.6) and ATPS (14/14% w/w PEG3350/potassium phosphate), determined by HPLC analysis of converted D-ABA.

100% conversion of D-ABA was achieved when the reaction was performed by the native form of the enzyme both in the buffer and in the ATPS. The PEGylated enzyme was less active, 55% D-ABA was converted in the ATPS and only 32% in the buffer. Surprisingly the PEGylated enzyme was more active in the ATPS than in a buffer.

3.3.4.4. Stability of PEGylated enzyme

PEG maleimide was stored in the presence of phosphorus pentoxide in a freezer as for stability it must be stored under anhydrous conditions. Each time a portion of MPEG-Mal was taken, the package was refilled with fresh phosphorus pentoxide. Therefore, if a larger amount of the enzyme was PEGylated and stored in the PEGylated form, this would be more convenient, provided the PEGylated enzyme was stable on storage.

Therefore, the stability of the PEGylated enzyme was tested. The activity of the stored PEGylated enzyme (23 days at +4°C) was compared with the freshly PEGylated one. Moreover, for comparison, the activity of the non-PEGylated enzyme was also determined.

The activity, determined by direct HPLC analysis of converted D-ABA, was expressed as % D-ABA converted by the enzyme within the same time. Results are presented in Table 24. The freshly PEGylated enzyme was able to convert nearly twice more D-ABA (57%) than the 23 days old solution of PEGylated one (29%). The native (non-PEGylated) enzyme was even more active (79% converted D-ABA). These results show that PEGylation not only decreases the enzyme activity but also its stability.

Subsequently it was found that the enzyme lost activity even if stored in an aqueous or a buffer solution. Thus it was not clear if the PEGylated enzyme lost its activity as a consequence of PEGylation or because it was stored in a buffer solution. However, the conclusion was that PEGylated enzyme could not be stored at 4°C for longer than a few hours and must therefore be made fresh each day.

Slavica *et al*[155] reported that the PEGylated DAAO could be stored at -25°C for several weeks without detectable loss of activity. However, in the current project, in contrast to the work of Slavica *et al*, the excess of MPEG-Mal was not removed. This might have been the reason for further instability of DAAO. At +4°C further PEGylation of the enzyme at Cys298, which is a part of the catalytic centre of the enzyme, might have happened. It has been proven[156] that PEGylation of this amino acid inactivates the enzyme.

			Peak Area [mVs]		D-ABA converted [%]	Mean conversion [%]
			L-ABA	D-ABA		
Native enzyme	Control	Rep. 1	141	31	78	79
		Rep. 2	147	29	80	
PEGylated enzyme	Freshly PEGylated	Rep. 1	117	51	56	57
		Rep. 2	119	52	57	
	Stored 23 days	Rep. 1	105	75	29	29
		Rep. 2	101	73	28	

Table 24 Stability of the PEGylated enzyme determined by HPLC analysis of converted D-ABA.

3.3.4.5. Effect of salt type on the PEGylated enzyme

The chiral analysis of ABA is described in section 2.5.1.2b. It was shown (2.5.1.2c) that the presence of ammonium sulphate, the salt component of ATPS used for the first CCC-bioreactor experiment (3.3.1.4), affects this assay. Therefore, the activity and stability of the PEGylated enzyme was determined in ATPS made of PEG3350 and five different salts (potassium phosphate, potassium citrate, sodium phosphate, sodium citrate and sodium sulphate), which were known to be compatible with the assay (2.5.1.2c).

The initial enzyme activity was determined at time 0, just after the enzyme was added to phase systems, and after 2h of enzyme incubation in ATPS. To determine the initial enzyme activity, 20µL of a substrate solution (1.66mg D/L-ABA) was added to 1.9ml of each ATPS. Next 100µL of PEGylated enzyme solution was added. Closed test tubes were placed on the rotating wheel with both phases being mixed for 10 min. After the elapsed time, the reaction was stopped by derivatisation of 50µL of sample with the mixture of orthophthalaldehyde and N-Isobutyryl-L-cysteine. The sample was analysed on HPLC.

To determine the enzyme activity after 2h, 100µL of PEGylated enzyme solution was added to 1.9ml ATPS and the samples were incubated for 2h on the rotary wheel. Then 20µL of a substrate solution (1.66mg D/L-ABA) was added to initiate the enzymatic reaction which was continued for 10min. Then the reaction was stopped by derivatisation of 50µL of sample for ABA (section 2.5.1.2d), which was analysed on HPLC.

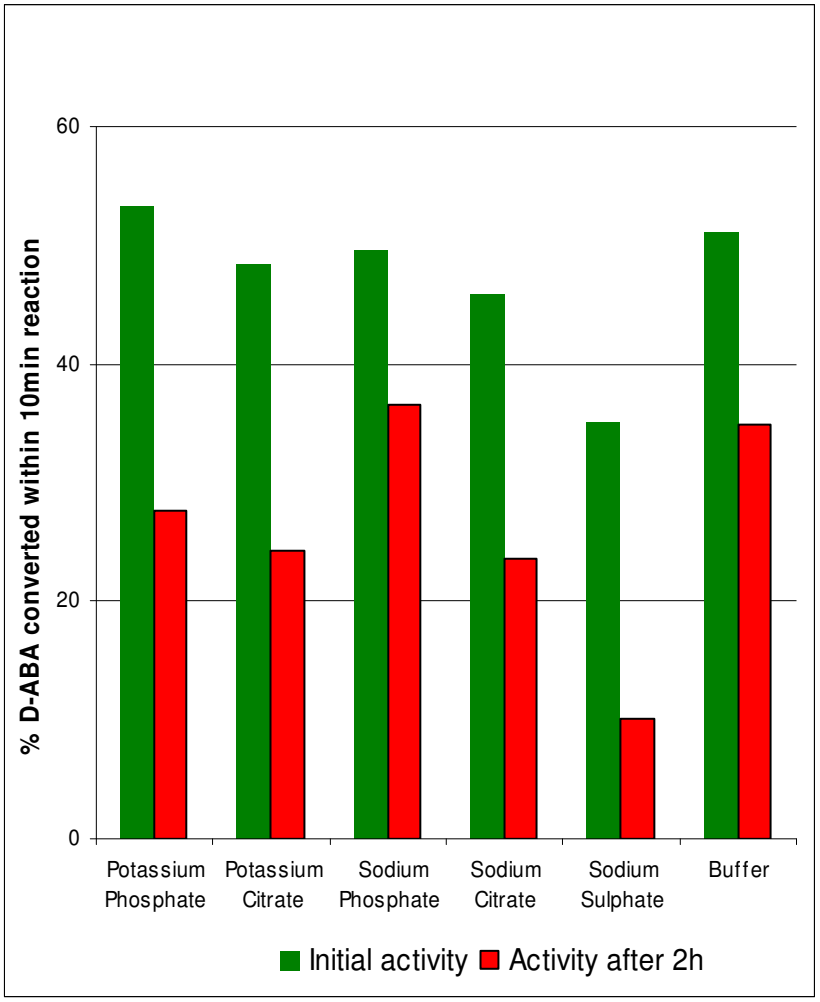


Figure 48 The initial PEGylated DAAO activity and the activity after 2h in ATPS 14/14% PEG3350 and a salt, determined by HPLC. The activity is expressed as a % of D-ABA converted within 10min of the enzymatic reaction.

The initial activity (green bar Figure 48) is the highest (53% converted D-ABA) for potassium phosphate and the lowest for the sodium sulphate (35% converted D-ABA). Based on the activity after 2h (red bar Figure 48) the enzyme is most stable in the presence of sodium phosphate (37% converted D-ABA) and the least stable in sodium sulphate (10% converted D-ABA).

The next stage was determining the distribution ratio of the PEGylated enzyme, DL-ABA and KBA in ATPS made of PEG3350 and the 5 chosen salts. Only 4 were tested as the ATPS 14/14% PEG3350/potassium citrate made a single phase when the enzyme and substrate solutions were added. That might have been caused by fact that ATPS PEG3350/potassium citrate at the concentration of 14/14% was close to the

binodal, the border of two phases. Therefore, a single phase was created when another component was added.

Salt type	ABA						KBA			Enzyme		
	L-ABA			D-ABA								
	Peak area		Ratio	Peak area		Ratio	Peak area		Ratio	Activity		Ratio
	UP	LP		UP	LP		UP	LP		UP	LP	
Potassium Phosphate	206	535	**0.39**	211	546	**0.39**	1466	660	**2.2**	0.190	0.006	**31.3**
Sodium Phosphate	315	968	**0.33**	324	983	**0.33**	1457	607	**2.4**	0.166	0.006	**27.6**
Sodium Citrate	433	878	**0.49**	448	901	**0.50**	1190	791	**1.5**	0.210	0.015	**14.2**
Sodium Sulphate	289	929	**0.31**	300	952	**0.31**	1322	594	**2.2**	0.156	0.006	**24.9**

Table 25 Distribution ratio of ABA, KBA and the PEGylated enzyme in the ATPS 14/14% w/w PEG3350/ salt. Both ABA and KBA were analysed on HPLC on C18 column according to methods described in section 2.5.1.2b for ABA and 2.5.1.4 for KBA. Peak areas [mVs] in upper (UP) and lower phase (LP) are given. The enzyme distribution ratio was determined based on its activity [abs/min] in both phases as determined by the colorimetric assay (2.5.2.1).

The distribution ratios of ABA and KBA, were within the range which was acceptable in all the tested systems (Table 25). Based on the distribution ratio and the enzyme stability, the best salt for the ATPS 14/14% PEG3350/salt was sodium phosphate where the enzyme stability was the highest. However, sodium phosphate was found to be susceptible to precipitation when used as part of ATPS 14/14% PEG3350. This could potentially block the coil of the CCC-bioreactor. For that reason potassium phosphate was selected as a salt for the ATPS.

3.3.4.6. CCC-bioreactor with PEG3350/potassium phosphate and PEGylated enzyme

To prove the compatibility of 14/14% PEG3350/ potassium phosphate with the CCC-bioreactor the experiment presented below was performed.

The enzyme was PEGylated prior to the experiment according to the procedure described in section 3.3.4.1.

2ml of DAAO obtained from Ingenza was incubated for 10 min at 4°C with 60 mg of MPEG-Mal in the presence of 8ml of 50mM sodium phosphate buffer, pH 7.5. This was then dissolved in 90ml of the upper, PEG-rich phase of 14/14% PEG3350/ potassium phosphate, used as the stationary phase. The mobile phase was pumped from head to tail of the coil at a flow rate of 0.5ml/min. 38.03mg DL-ABA was

injected onto the equilibrated CCC column. Fractions of the mobile phase eluted from CCC were analysed for ABA using the chiral derivatisation method (2.5.1.2b).

Figure 49 shows a fractogram reconstructed based on HPLC analysis of ABA. In the graph there are two sets of bars, which correspond to peak areas of the two enantiomers in each fraction, plotted against the elution time. In each fraction a peak area of D-ABA peak is smaller than a peak area of L-ABA, which is assumed to be caused by the enzymatic conversion of D-ABA. The total D-ABA conversion ratio was 22.8%. Although this is far from 100%, which is the requirement of the project, it demonstrates the potential of both the selected phase system as well as the CCC instrument used as a bioreactor.

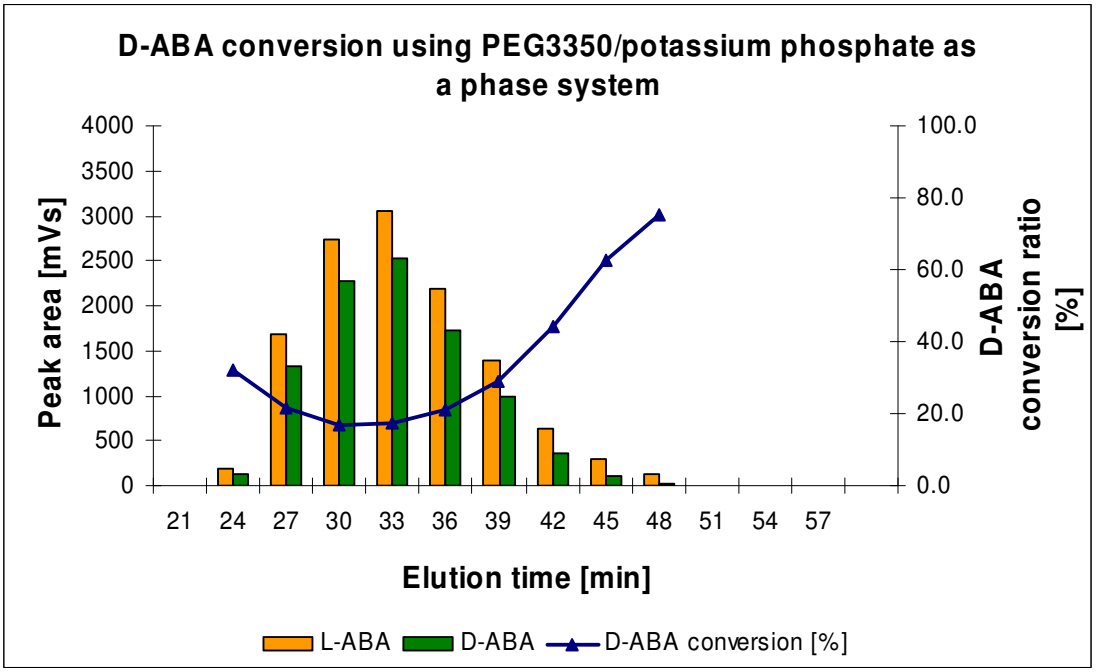


Figure 49 Elution profile of ABA from the CCC-bioreactor (Milli, coil volume 45.1ml ref. section 2.2.1). 38.06mg DL-ABA was injected onto the column. Rotational speed 1800rpm. Stationary phase displaced 19.5ml. The mobile phase was the lower phase of 14/14% PEG3350/ potassium phosphate (pH 7.5) pumped from head to tail direction at the flow rate 1.0ml/min. The stationary phase was the top phase with the PEGylated enzyme DAAO (10ml of the enzyme PEGylated enzyme dissolved in 90ml of the upper phase). The temperature was 20°C.

3.3.5. ATPS of PEG1000 with native enzyme

ATPS made of PEG1000 in concentration 14/14% was one of the two potential phase systems selected for the CCC-bioreactor (3.3.3). Section 3.3.1.4 presents the unsuccessful CCC-bioreactor with no bioconversion detected, using the ATPS made

of PEG1000 and ammonium sulphate as a phase system. However, the phase system used in that experiment was employed without consideration of the enzyme stability, which was subsequently found to be a critical issue in the process. However, different salts had been found to affect the enzyme stability at different rates so PEG1000 was revised in the light of this new knowledge and with the aim of optimising enzyme stability for the experiment.

3.3.5.1. Overview of section

From Table 22 it is known that for the desirable enzyme partitioning the concentration of ATPS made of PEG1000 should be not higher than 14/14%. Based on the studies presented in the next sections, ammonium phosphate provided better enzyme partitioning as well as stability (3.3.5.2) than ammonium sulphate, which was used for the first CCC-bioreactor (section 3.3.1.4). It was also discovered that the PEGylated enzyme was more stable in the presence of phosphates, rather than in the presence of the sulphate anion (3.3.4.5). It has to be mentioned that ATPS made of PEG1000 does not require the PEGylated enzyme.

The ammonium cation, which cannot be used with the chiral detection method of ABA (2.5.1.2c), was therefore replaced by the potassium cation. Furthermore, the sodium cation was also rejected as, at the concentration used in ATPS, sodium phosphate is susceptible to precipitation.

3.3.5.2. Identification of optimum salt for PEG1000 ATPS

To identify a suitable salt anion, two experiments were performed to determine the difference in the enzyme partitioning and stability in the presence of sulphate and phosphate salts. Previously (section 3.3.1.4) sulphate salts had been used with the CCC-bioreactor but data form the PEGylation experiments (section 3.3.4.5) suggested that phosphate salts were more suitable. Therefore these two salt anions were compared as follows.

The enzyme distribution ratio was determined in the following ATPS:

- 14/14 % (w/w) PEG1000/ ammonium sulphate
- 14/14 % (w/w) PEG1000/ ammonium phosphate

100µl of the enzyme supernatant was dissolved in 5ml of the upper and 5ml of the lower phase. The phases were mixed and left for 10min to allow them to settle. The

enzyme activity was then determined in the upper and in the lower phase in the colorimetric assay. A D-value was determined by dividing the enzyme activity (absorbance/min) in the upper phase by the enzyme activity in the lower phase.

In both cases the enzyme partitioned into the upper, PEG-rich phase (Table 26). The distribution ratio was higher when the phosphate salt was used, therefore ATPS made of that salt would better suit the CCC-bioreactor, as the enzyme has to remain immobilised in the stationary phase of the CCC-bioreactor purely by distribution for the process to operate effectively.

PEG1000/phosphate 14/14%			PEG1000/sulphate 14/14%		
Activity		D-value	Activity		D-value
Upper phase	Lower phase		Upper phase	Lower phase	
0.128	0.006	**20.5**	0.099	0.015	**6.7**
0.141	0.014	**10.3**	0.107	0.015	**6.9**

Table 26 DAAO distribution ratio in ATPS 14/14% PEG1000/ ammonium phosphate and 14/14% PEG1000/ ammonium sulphate.

In the same phase systems, the enzyme stability was tested as follows: 50µl of the enzyme supernatant was added separately to 4950µl of the upper and the lower phases the tested phase systems. Then the enzyme activity was determined by the colorimetric assay (2.5.2.1) immediately after mixing the enzyme with the phase system (time 0) and subsequently after 1 and 2 hours of incubation at room temperature (22°C).

Figure 50 shows the results of enzyme stability in 14/14% w/w PEG1000/ammonium sulphate and in 14/14% w/w PEG1000/ammonium phosphate. It was found that the enzyme lost part of its activity in the tested ATPS. After 2h the enzyme lost 48% of its initial activity in the upper phase of 14/14% PEG1000/ammonium phosphate and 59% in the upper phase of 14/14% PEG1000/ammonium sulphate. Therefore, the DAAO stability was slightly better when the phosphate salt was used rather than the sulphate one.

This experiment confirmed that the enzyme lost activity quicker in an upper PEG-rich phase, compared to a bottom, salt-rich phase. That was true for the both examined salts. Despite the enzyme being more stable in the lower phases, they could not be used as the stationary phase in the CCC-bioreactor due to partitioning of the enzyme

into the PEG-rich phase. In addition, it is more difficult to retain the salt-rich phase in CCC. Figure 73 (section 5.3.1) shows that there was more stationary phase displaced, which means poorer retention, when the lower-salt rich phase was used as the stationary phase. Another reason to use the salt-rich phase as the mobile phase is that the extraction of L-ABA is easier than from the upper-PEG rich phase.

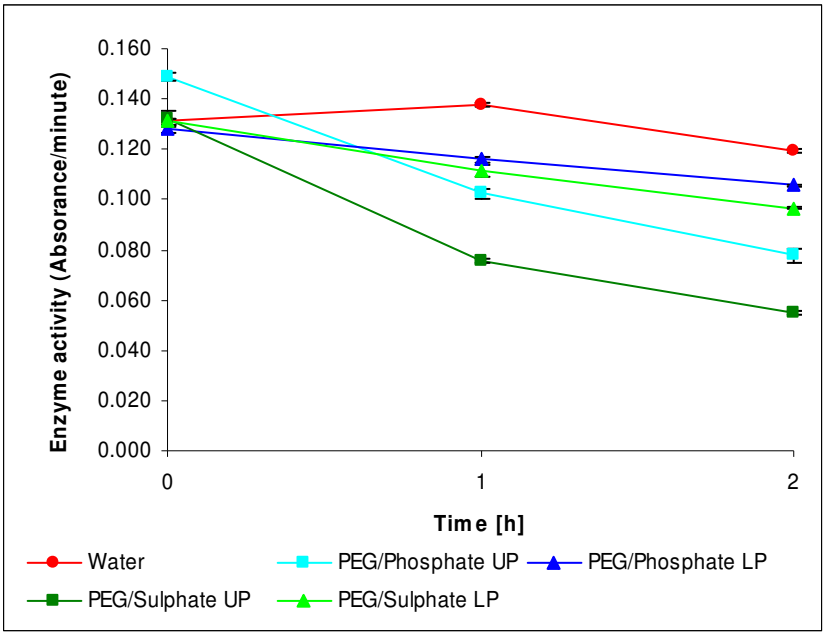


Figure 50 Stability of DAAO in 14/14% PEG1000/ammonium sulphate and 14/14% PEG1000/ammonium phosphate. 50µl of the enzyme supernatant was dissolved in 4950µl of each phase separately. Samples were incubated at room temperature (22°C). Enzyme activity was determined by the colorimetric assay at the time 0, 1 and 2 hours.

3.3.5.3. CCC-bioreactor using PEG1000/ potassium phosphate and native enzyme

The experiment was performed to evaluate ATPS made of PEG 1000 as a phase system for the CCC-bioreactor. All experimental conditions, apart from the tested phase system and the form of the enzyme, were the same as in experiment presented in section 3.3.4.6. Since the tested ATPS was made based on PEG1000, no PEGylation of the enzyme was required. 2ml of the enzyme was dissolved directly in 98ml of the upper phase so the concentration of the enzyme in the phase system was the same as in the experiment presented in section 3.3.4.6.

Figure 51 shows the fractogram obtained based on HPLC analysis of ABA eluted from the CCC-bioreactor using 14/14% PEG1000/potassium phosphate as the phase system. The total conversion of D-ABA was 22.6%, which was very close to that

obtained with PEG3550 and the PEGylated enzyme (22.8%). Although this is a long way from the ideal conversion rate of 100%, this figure of 22.6% was obtained using a CCC-bioreactor whose operating parameters had not been optimised and it therefore only shows compatibility of the tested phase system for the CCC-bioreactor. Optimisation of the CCC-bioreactor operating parameters is the subject of Chapter 4.

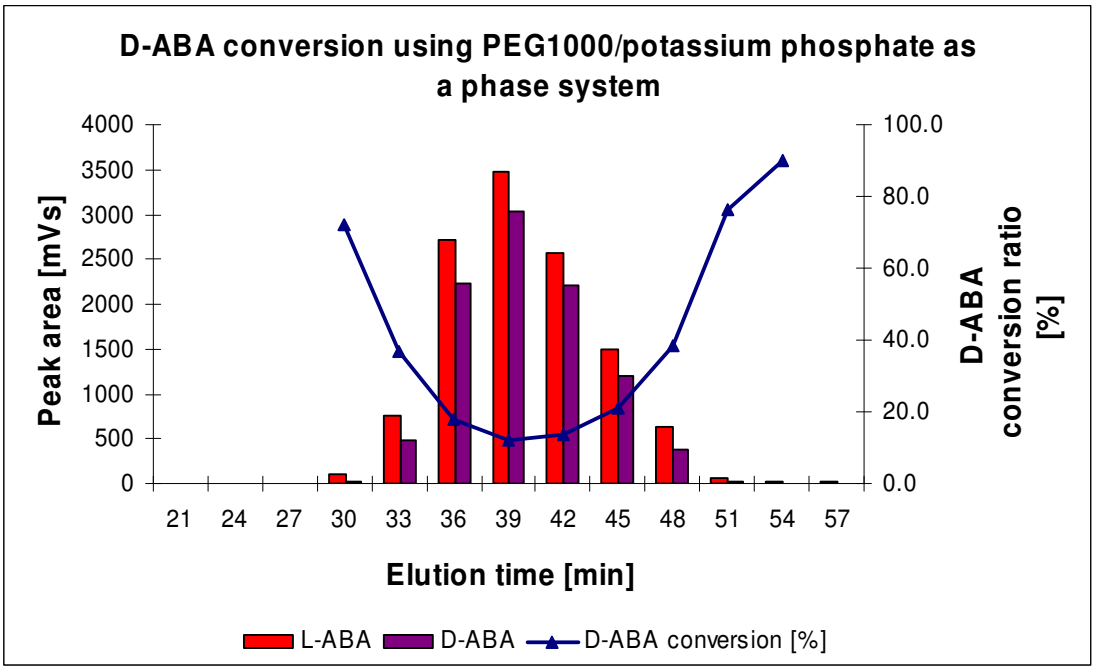


Figure 51 Elution profile of ABA from the CCC-bioreactor (Milli, coil volume 45.1ml ref. section 2.2.1) using 14/14% PEG1000/ potassium phosphate as a phase system. 38.08mg DL-ABA was injected onto the column. Rotational speed 1800rpm. Stationary phase displaced 25ml. The mobile phase was the lower phase, pumped from head to tail direction at the flow rate 1.0ml/min. The stationary phase was the top phase with enzyme DAAO (2ml of the enzyme dissolved in 98ml of the upper phase). The temperature was 20°C.

3.3.6. Comparing the two ATPS systems made of PEG1000 and PEG3350

The two two-phase systems, 14/14% w/w PEG3350/potassium phosphate and 14/14% w/w PEG1000/potassium phosphate, were compared in terms of their ability to maintain the enzyme activity. For ATPS made of PEG1000 the native form of the enzyme (20µl) was used, whereas for the ATPS made of PEG3350 100µl of the PEGylated form (containing 20µl of the native form) was used. The experiment was done in triplicate. Table 27 shows volumes of the enzyme solution and phases used for the experiment.

	Volume [µl]			
	Native enzyme		PEGylated enzyme	
	PEG1000/potassium phosphate	Buffer	PEG3350/potassium phosphate	Buffer
Enzyme*	20	20	100	100
UP	980	0	900	0
LP	1000	0	1000	0
Buffer	0	1980	0	1900
Total	2000	2000	2000	2000

Table 27 Volume (µl) of the enzyme, upper phase (UP), lower phase (LP) and buffer (50mM potassium phosphate pH 7.8).

* the enzyme was used in the native form for PEG1000 ATPS, and the PEGylated form for the ATPS made of PEG3350. The volume of the enzyme supernatant added in both cases was 20µl

The enzyme was incubated with the tested phase systems on the rotating wheel for 2 and 4 hours. After that time, 20µl the substrate DL-ABA at the concentration of 150mg/ml was added directly to a phase system. The reaction was stopped after 10min by derivatising 50µl of a sample using the ABA chiral derivatisation method, described in section 2.5.1.2d. Results are presented in Figure 52.

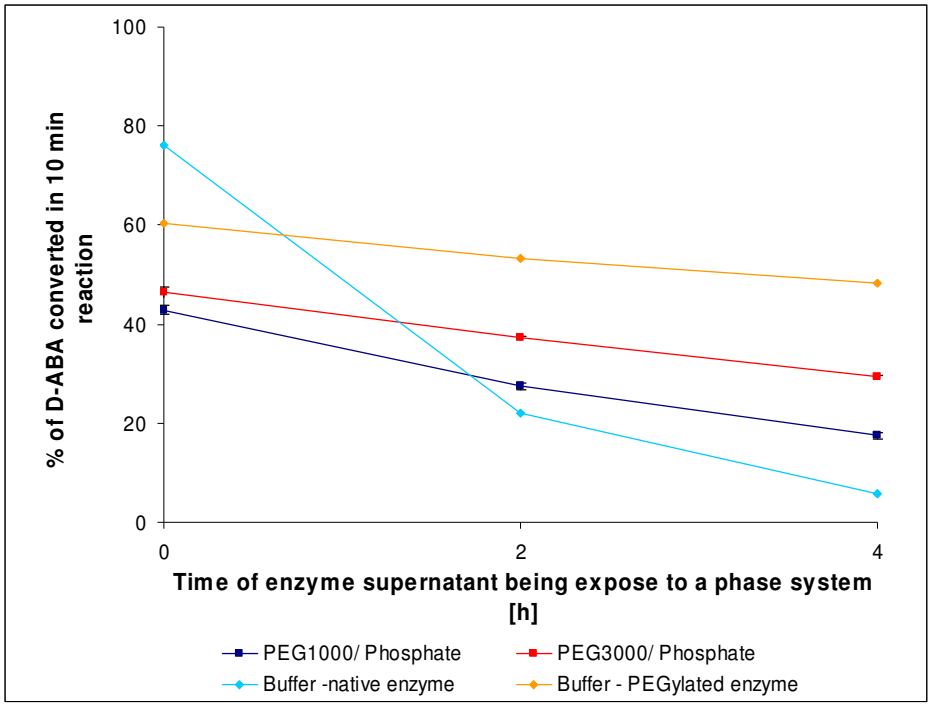


Figure 52 Comparison between the two-phase systems 14/14 PEG1000/potassium phosphate (native enzyme) and 14/14 PEG3350/potassium phosphate (PEGylated enzyme) performed by HPLC measuring converted D-ABA.

Unfortunately, the results showed that both the enzyme activity and stability are better in 14/14% w/w PEG3350/potassium phosphate. In 14/14 % w/w PEG1000/potassium phosphate the enzyme was less active and less stable. However, despite the slightly lower activity it was decided to do a CCC-bioreactor with PEG1000/potassium phosphate and the native enzyme as a comparison to the previous bioreactor run using PEG3350/potassium phosphate and the PEGylated enzyme.

3.3.7. Conclusions on ATPS as solvent system for the CCC-bioreactor

Experiments presented in sections 3.3.4.6 and 3.3.5.3 show the potential of both phase systems to be applied for the CCC-bioreactor. Both phases were tested in the same conditions. The bioconversion of D-ABA was the same for both phases (22.8%, with PEG3350; 3.3.4.6 and 22.6% with PEG1000; 3.3.5.3).

Due to a favourable partitioning of the enzyme, the main advantage of using 14/14% w/w PEG1000/potassium phosphate over the alternative 14/14% PEG3350/potassium phosphate is that this phase system does not require the use of the PEGylated enzyme. Therefore, because of the simplicity and the fact that PEGylating of the enzyme might cause an extra variable, affecting reproducibility of other experiments, the second solvent system, 14/14% w/w PEG1000/ potassium phosphate, was selected for optimising the CCC-bioreactor, which is presented in chapter 4.

Before then, the potential for using a completely different, non-ATPS phase system was studied. Aqueous-organic systems are studied in section 3.4 and aqueous-ionic liquid systems in section 3.5.

3.4. Aqueous-organic systems as solvent systems for the CCC-bioreactor

3.4.1. Introduction

It has been assumed that the best solvent system suitable for the CCC-bioreactor was an aqueous two-phase system (ATPS). However, using this type of system caused challenges, such as problems with enzyme stability and distribution ratio, product and substrate analysis. Moreover ATPS can be very viscous and cause problems with the pumping system and the viscosity also results in poor stationary phase retention, requiring the use of a wide bore CCC coil. Also, poor mixing of the phases is suspected due to the high viscosity of the PEG and this results in slow mass transfer of molecules between the phases.

To overcome all, or at least some, of these problems, the possibility of using organic solvents was tested. There would be a lot of advantages of using organic systems. Organic solvents are easy to make, cheaper and more suitable for CCC because of their low viscosity. The product can be easily isolated from a fraction by evaporating the solvent. Moreover, separations of compounds and the behaviour of aqueous-organic phase systems in CCC has been far better studied than with ATPS.

The deciding factor in using organic solvents in a CCC-bioreactor is enzyme activity and stability. For the vast majority of enzymes, an aqueous environment is the most natural and they demonstrate the highest activity there. However the literature shows numerous enzymatic reactions performed in even a totally anhydrous environment[157,158,159].

This section investigates the testing of D-amino acid oxidase (DAAO) activity in 36 aqueous-organic two-phase systems.

3.4.2. Experimental

Materials

Enzyme D-amino acid oxidase (DAAO) was received from Ingenza. The received crude material was centrifuged on the Micro Centaur centrifuge at 10,000 rpm for 5min. For all subsequent analysis, the enzyme supernatant was taken.

The activity of D-amino acid oxidase was tested in 36 organic solvents systems developed by Dr Ian Garrard for the collaborative project on "Anti-angiogenic activities of the volatile oil of *Angelica sinensis*"[160]. These 36 systems represented a wide range of two phase organic systems commonly used for the CCC separation of natural compounds. All the 36 solvent system were made by means of a liquid-handling robot (6.4.3.3), using 12 organic solvents and water. The compositions of all 36 solvent systems are presented Table 28.

Determination of enzyme activity in organic systems

To each 5 ml test tube containing 2ml of each phase system, 10µl D/L-ABA (133mg/ml) water solution was added. Next the enzymatic reaction was initiated by adding 20µl enzyme supernatant to every test tube with a 30s interval. A rotating wheel provided continuous phase mixing during the reaction. The bioconversion was stopped after 30 min (timer) by derivatisation of the sample. Derivatised samples were analysed by HPLC.

The enzyme activity was expressed as a percent of unreacted D-ABA that remained after the 30 minute reaction. L-ABA was used as an internal standard, the amount of which was assumed to be identical to the initial amount of D-ABA.

Sample analysis

50µl sample (a mixture of upper and lower phase) was derivatised with 150µl mixture of ortho-phthalaldehyde and N-isobutyryl-L-cysteine in 0.4M potassium borate buffer pH 10 and after 90s diluted to 1ml with water. As mentioned above, the L-ABA present in each sample was used as an internal standard. Thus it was not important how complete the derivatisation process was as both forms D- and L- are derivatised to the same degree. Also an accurate mixing of both phases before taking a sample does not play any important role as both forms of the amino acid are present in equal amounts in any given phase.

3.4.3. Result and Discussion

The enzyme activity was expressed as percent of converted D-ABA after a 30 min reaction (Table 28). The higher the value, the higher was the enzyme activity in a system, as more D-ABA must have been converted. In the last column enzyme activity is shown using different colours. A green colour shows very high enzyme activity, whereas red indicates very low.

As expected, the enzyme shows very low activity in most of the tested systems (red colour). The highest conversion was obtained for systems No 4 (water/ethyl acetate) and 27 (water/tert-butyl methyl ether). These two systems consist of water and a nonpolar solvent. According to Kral *et al*[54] (2002), solvents with a log P>3, such as xylene (log P = 3.1) or hexane (log P = 3.9) are less deactivating than those with a low log P, such as ethanol (log P = -0.24). A big difference in polarity of these systems might keep the two phases completely separated, the lower phase consisting only of water whereas the upper of nonpolar organic solvent. The enzyme, being a naturally hydrophilic molecule, is totally distributed in the water phase, which has limited contact with the strongly nonpolar organic solvent (the contact is limited only to the interfacial area). It is interesting to note that the enzyme shows relatively high conversion in the totally organic environment consisting of heptane and acetonitrile (No. 15). However the peak areas for both D- and L-ABA are very low compared with the other systems. This is because of the very low solubility of ABA in organic solvents.

System No.	Solvent [ul]													Peak Area [mVs]		D-ABA conversion [%]
	Heptane	EtOAc	MeOH	Butanol	Water	ACN	Toluene	Acetic Acid	Acetone	tBME	DCM	Ethanol	Propanol	L-ABA	D-ABA	
1	0	0	0	1000	1000	0	0	0	0	0	0	0	0	513	486	5
2	0	400	0	600	1000	0	0	0	0	0	0	0	0	518	493	5
3	0	800	0	200	1000	0	0	0	0	0	0	0	0	477	453	5
4	0	1000	0	0	1000	0	0	0	0	0	0	0	0	686	192	72
5	145	855	145	0	855	0	0	0	0	0	0	0	0	620	553	11
6	250	750	250	0	750	0	0	0	0	0	0	0	0	574	552	4
7	400	600	400	0	600	0	0	0	0	0	0	0	0	572	553	3
8	545	455	545	0	455	0	0	0	0	0	0	0	0	539	522	3
9	715	285	715	0	285	0	0	0	0	0	0	0	0	647	618	5
10	835	165	835	0	165	0	0	0	0	0	0	0	0	644	621	4
11	950	50	950	0	50	0	0	0	0	0	0	0	0	588	559	5
12	1000	0	1000	0	0	0	0	0	0	0	0	0	0	676	646	4
13	1000	0	900	0	0	100	0	0	0	0	0	0	0	608	578	5
14	1000	0	600	0	0	400	0	0	0	0	0	0	0	913	874	4
15	1000	0	0	0	0	1000	0	0	0	0	0	0	0	44	17	62
16	1000	0	0	0	0	900	100	0	0	0	0	0	0	55	27	50
17	1000	0	0	0	0	600	400	0	0	0	0	0	0	122	111	9
18	0	0	0	950	950	0	0	100	0	0	0	0	0	177	157	11
19	0	0	0	900	900	0	0	200	0	0	0	0	0	64	49	24
20	800	0	560	0	240	0	0	0	0	0	400	0	0	452	426	6
21	0	0	0	400	1000	200	0	0	0	400	0	0	0	550	528	4
22	625	250	500	0	0	625	0	0	0	0	0	0	0	327	309	5
23	0	0	0	842	1052.5	0	0	0	105.5	0	0	0	0	534	515	4
24	333	333.5	0	0	333.5	0	0	0	1000	0	0	0	0	541	442	18
25	1000	0	800	0	0	0	0	0	200	0	0	0	0	571	559	2
26	0	0	0	400	1000	200	0	0	0	400	0	0	0	535	518	3
27	0	0	0	0	1000	0	0	0	0	1000	0	0	0	452	0	100
28	0	0	800	0	533.5	0	0	0	0	0	666.5	0	0	346	335	3
29	1000	0	0	0	0	800	0	0	0	0	200	0	0	260	210	19
30	750	0	0	0	625	0	0	0	0	0	0	625	0	677	653	4
31	513	513	0	0	307.5	0	0	0	0	0	0	666.5	0	574	550	4
32	0	800	0	0	800	0	0	0	0	0	0	400	0	518	498	4
33	0	500	0	800	600	0	0	0	0	0	0	100	0	474	455	4
34	0	0	0	889	889	0	0	0	0	0	0	222	0	508	484	5
35	1000	0	0	0	750	0	0	0	0	0	0	0	250	575	552	4
36	1143	0	457	0	114.5	0	0	0	0	0	0	0	285.5	778	752	3
Water					2000									550	0	100
Water					2000									568	0	100

D-ABA conversion [%]

< 10

10-29

30-50

> 50

Table 28 Conversion of D-ABA by DAAO in a range of aqueous-organic two phase systems. The table shows the composition of 36 organic two-phase systems which are combinations of 12 organic solvents and water. In two columns are shown peak areas of D- and L-ABA derivatised and analysed on the HPLC after 30 min bioconversion. In the last column percent of unreacted D-ABA is given. The conversion ratio is marked with different colours. In green are marked systems with a very high conversion ratio - less than 50% D-ABA stayed unconverted. Whereas, in red colour are shown systems in which the conversion was very low, more than 90% D-ABA stayed unreacted

The experiment was repeated for 7 solvent systems in which the D-ABA conversion was greater then 50% (Table 28). Results are presented in Table 29.

System No.	Solvent [ul]											Peak Area [mVs]		D-ABA conversion [%]
	Heptane	EtOAc	Butanol	Water	ACN	Toluene	Acetic Acid	Acetone	DCM	tBME		L-ABA	D-ABA	
4	0	1000	0	1000	0	0	0	0	0	0		553	171	69
15	1000	0	0	0	1000	0	0	0	0	0		42	18	56
16	1000	0	0	0	900	100	0	0	0	0		946	931	2
19	0	0	900	900	0	0	200	0	0	0		40	36	10
24	333	333.5	0	333.5	0	0	0	1000	0	0		277	232	17
27	0	0	0	1000	0	0	0	0	0	1000		855	0	100
29	1000	0	0	0	800	0	0	0	200	0		191	162	15
Water	0	0	0	2000	0	0	0	0	0	0		593	0	100

Table 29 The composition of 7 aqueous-organic and organic-organic two phase solvent made of 9 organic solvents and water. The volume of each solvent is given in µL. The total volume of each phase was 2ml. In the last column is given a conversion ratio of D-ABA after 30min. of incubation with DAAO

The highest conversion ratio (100%) was achieved in the system No.27, composed of water and tBME. Good conversions were obtained in systems No. 4 and 15, 69% and 56%, respectively. In contrast to the experiment presented in Table 28, this time in systems No. 16 and 19 the conversion ratios were only 2 and 10%, respectively. The reason of such a difference is unknown but it shows a lack of robustness of these phase systems and therefore they were rejected.

The next tested parameter was the ABA partitioning. It was tested in the 3 solvent systems; 4, 15 and 27, selected based on the enzyme activity In those 3 systems, the D-ABA conversion was >50% as expressed in Table 29. The partitioning results are presented below in Table 30.

System No.	Peak area [mVs]				Distribution ratio	
	Upper phase		Lower phase			
	L-ABA	D-ABA	L-ABA	D-ABA	L-ABA	D-ABA
4	0	0	2298	2368	**0.00**	**0.00**
15	24	27	61	63	**0.39**	**0.42**
27	0	0	2270	2350	**0.00**	**0.00**

Table 30 Distribution ratio of ABA was tested in 3 solvent systems (No4 EtOAc-water, No15 heptane-ACN and No27 water-tBME) by HPLC.

As expected ABA, which is a very polar compound, totally partitions into the lower, water phase in systems No 4 and 27. As previously mentioned, it is possible that also the enzyme stayed active in these systems only because it totally partitioned into the water phase, not having any contact with the water-immiscible organic phase.

ABA was equally distributed only in the system No 15 (heptane/ACN) D=0.4. However the peak area is very small, which suggests poor solubility of the tested compound in organic solvents. A very polar ABA might have partitioned into the interface area or even precipitated, as both components of the system heptane and acetonitrile are nonpolar.

However, the results suggest that in the aqueous-organic two-phase systems, where the ABA partitioned completely to the aqueous phase, one possible way to drive ABA from the lower-water phase to the upper-organic phase would be applying a third nonpolar but water miscible solvent, such as acetonitrile. This solvent, being miscible with both water and most organic solvents, would reduce the difference in polarity between an extremely polar water phase and a nonpolar organic phase.

Moreover acetonitrile seems to be the best choice for this third solvent as the enzyme was active in system No 15, composed of acetonitrile and heptane (Table 29).

Therefore, system No 27 was modified by replacing a part of water and tBME by acetonitrile. The enzyme activity was determined by measuring D-ABA conversion ratio after 30min. Results are given in the Table 31.

Results show that any presence of acetonitrile in the solvent system makes the enzyme totally inactive. A full D-ABA conversion took place only in case of the control (water) and system No 27 (water and tBME). Lack of any D-ABA conversion in the presence of acetonitrile may be explained by the fact that the solvent makes tBME more polar, thus more accessible to the enzyme, and therefore inactivating it.

System No.	Solvent volume			Peak Area [mVs]		D-ABA converted
	Water	tBME	ACN	L-ABA	D-ABA	
27 A	1000	1000	0	364	0	**100**
27 B	816	816	367	709	724	**0**
27 C	690	690	621	714	734	**0**
27 D	597	597	806	578	595	**0**
27 E	526	526	947	514	525	**0**
Water	2000	0	0	611	0	**100**

Table 31 DAAO activity in two-phase systems composed of water tBME and ACN determined by HPLC analysis of converted D-ABA.

It should be noted that the enzyme activity was performed by determining its substrate conversion ratio. If the enzyme did not show any activity, this could be caused by its inactivation/inhibition or by the lack of available substrate which is de facto insoluble in organic solvents.

3.4.4. Conclusions on aqueous-organic systems as solvent systems for the CCC-bioreactor

Conversion of D-ABA was not possible in most of the tested organic systems. However, a 100% conversion was achieved in two of the tested water/organic solvents systems: water/heptane and water/tBME. The biggest obstacle to using a water/organic solvent system in the CCC-bioreactor comes from the unfavourable distribution ratio of the substrate and in all probability, the product of the reaction as well. Thus of the tested organic systems, none of them totally satisfied all the requirements presented in the Introduction (3.1).

3.5. Ionic liquids as solvent systems for the CCC-bioreactor

3.5.1. Introduction

Ionic liquids (IL) are salts that do not crystallise at room temperature. Most of them are not soluble with water, with which these form two-phase systems. Therefore their liquid states make them possible candidates as solvents in CCC.

Ionic liquids have been tested for use as a main component of a solvent for CCC separation. Berthod and Carda-Broch[161] (2004) tested a phase system based on an IL, 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6] and water as a phase system for CCC. The study of the partitioning of aromatic solutes between water and [BMIM][PF_6] demonstrated the solvent capacity of IL. However, it was found that due to a very high viscosity of ionic liquids at room temperature, they cannot be used directly as a solvent system in countercurrent chromatography. Therefore, acetonitrile was the third component added in order to reduce the very high viscosity of [BMIM][PF_6]. A composition of 40:20:40% w/w water-acetonitrile-[BMIM][PF_6] was proposed as a phase system for CCC.

There has been reported the use of an IL for protein separation in CCC[162]. [BMIM][Cl] was used instead of PEG, to make an aqueous two-phase system with potassium dibasic phosphate (K_2HPO_4). There was no separation with the system as all four tested proteins were totally distributed in the IL-stationary phase. This behaviour would be ideal for the work on the CCC-bioreactor described in this thesis, with the enzyme totally distributed in an IL-stationary phase, as it would eliminate the problem of enzyme eluting with the mobile phase.

The activity and stability of free and immobilised D-amino acid oxidase (DAAO) has been tested in the presence of five alkylimidazolium based ionic liquids by Lutz-Wahl *et al* in 2006[163]. The immobilised enzyme was most active in the presence of [BMIM][BP_4] and [MMIM][$MMPO_4$], which however had the undesirable property of being miscible with water. For the two tested, water insoluble ILs, [BMIM][Tf_2N] and [BMIM][PF_6], the activity was reduced by 50%. The authors proposed mass transfer limitation as the explanation of the lower velocity of the reaction in water-insoluble ILs. However, the enzyme stability for the two water-insoluble ILs was relatively high (over 80% activity after 24h incubation at 30°C), compared with water

soluble ILs. This would be a great advantage of using IL for the CCC-bioreactor, considering that enzyme stability in the stationary phase is a challenge.

In this section the results of the enzymatic conversion of D-amino butyric acid in two-phase systems made of eight different ionic liquids are presented. These ILs were provided by Northern Ireland's Queen's University Ionic Liquids Laboratories (QUILL) for examination for use in CCC.

3.5.2. Experimental

Materials

Enzyme D-amino acid oxidase (DAAO) was received from Ingenza. The received crude material was centrifuged on the Micro Centaur centrifuge at 10,000 rpm for 5min. For all subsequent analysis the enzyme supernatant was taken.

The activity of D-amino acid oxidase (DAAO) was tested in 8 Ionic Liquids, obtained from QUILL:

1. [C6, 6, 6, 14P][NTf2]
2. [C6, 6, 6, 14P][N(CN)2]
3. [BMIM][PF6]
4. [BMIM][BF4]
5. [EMIM][NTf2]
6. [N,N,N,N-dimethyl-ethyl-methoxyethyl-ammonium][NTf2]
7. 1-allyl-3-methlyimidazolium][NTf2]
8. 1-methoxyethyl-3-methylimidazolium][NTf2]

The enzyme activity was tested in two-phase systems made of IL and 50mM sodium phosphate buffer pH 7.6.

Determination of enzyme activity in Ionic Liquid systems

To each 5 ml test tube containing 1ml of IL, and 0.98 ml buffer, 20µl D/L-ABA (100mg/ml) water solution was added. To confirm that the presence of IL does not affect the ratio of L- to D-ABA peak area, both phases were mixed and after phase separation, 50µl of the buffer phase was derivatised and analysed on HPLC.

The enzymatic reaction was then initiated by adding 20µl enzyme supernatant to every test tube with a 30s interval. A rotating wheel provided continuous phase mixing during the reaction at room temperature (25°C). The bioconversion was stopped after 30 min (timer) by derivatisation of each sample with the mixture of ortho-phthalaldehyde (OPA) and N-isobutyryl-L-cysteine in 0.4M potassium borate buffer. Derivatised samples were analysed on HPLC. As a control, 20µl D/L ABA (100mg/ml) was dissolved in 0.98ml buffer.

Sample analysis

Before adding the enzyme, 50µl of buffer phase was premixed with 100µl 0.4M potassium borate buffer, pH 10, and next derivatised with 50µl OPA/ N-isobutyryl-L-cysteine solution. After 90s, the samples were diluted with 800µl of water and analysed on HPLC using a C18 column.

In order to stop the enzymatic reaction 50µl of buffer phase was mixed with OPA/ N-isobutyryl-L-cysteine solution premixed with 0.4M potassium borate buffer, pH 10. As mentioned above, the L-ABA present in each sample was used as an internal standard. Thus it was not important how precise the derivatisation process was as both forms D- and L- are derivatised to the same degree.

3.5.3. Results and Discussion

The enzyme activity was expressed as a percent of converted D-ABA after the 30 min. reaction. L-ABA was used as an internal standard, the amount of which was assumed to be identical to the initial amount of D-ABA.

Ionic liquid name	Before enzyme was added			After 30min incubation with the enzyme			Upper phase	Lower phase
	Peak Area [mVs]		Ratio of D-ABA to L-ABA [%]	Peak Area [mVs]		D-ABA conversion [%]		
	L-ABA	D-ABA		L-ABA	D-ABA			
[C6, 6, 6, 14P][NTf2]	1731	1762	**-1.8**	1637	1234	**25**	Buffer	IL
[C6, 6, 6, 14P][N(CN)2]	1705	1718	**-0.8**	1326	0	**100**	IL	Buffer
[BMIM][PF6]	1823	1853	**-1.6**	1741	588	**66**	Buffer	IL
[BMIM][BF4]	879	878	**0.2**	837	726	**13**	miscible	
[EMIM][NTf2]	1832	1864	**-1.8**	1727	1343	**22**	Buffer	IL
[N,N,N,N-dimethyl-ethyl-methoxyethyl-ammonium][NTf2]	1870	1903	**-1.7**	1767	901	**49**	Buffer	IL
1-allyl-3-methlyimidazolium][NTf2]	1873	1906	**-1.7**	1739	674	**61**	Buffer	IL
1-methoxyethyl-3-methylimidazolium][NTf2]	2057	2090	**-1.6**	1923	354	**82**	Buffer	IL
Control - buffer	1816	1849	**-1.9**	1766	0	**100**	-	-

D-ABA conversion [%]
< 30
30-59
60-79
> 80

Table 32 DAAO activity tested in two-phase systems based on ionic liquids and 50mM sodium phosphate buffer, pH 7.6. The activity was expressed as percent of D-ABA converted within 30 min based on the ratio of D-ABA and L-ABA analysed on HPLC. Peak area of D- and L-ABA was determined twice for each sample: before the enzyme was added and 30min after the enzyme addition.

[BMIM][BF4] made a single phase with the buffer. All the other ILs were immiscible with water, making two-phase systems. Apart from [C6, 6, 6, 14P] [N(CN)2] which was the main component of the upper phase, the rest of the water immiscible ILs formed the lower phase of a system.

Before adding the enzyme, the ratio of area for both chiral forms of ABA was closely similar for all the tested IL (Column IV, Table 32). Within the error of HPLC integration, the presence of IL does not affect the ratio D-ABA to L-ABA. Thus the enzyme activity can be expressed as the ratio of the two peaks.

Column VII of the Table 32 shows the enzyme activity as % of D-ABA converted after the 30min reaction. There was some conversion in all eight tested systems. Enzyme was most active in [C6, 6, 6, 14P][N(CN)2], where 100% D-ABA were converted. The smallest activity (13% D-ABA converted) occurred for the [BMIM][BF4]/buffer system. As [BMIM][BF4] was totally miscible with water, it had a better contact with the enzyme and might have inactivated it or decreased its activity.

Lutz-Wahl *et al* [163] have demonstrated that DAAO activity depends on the ionic liquid concentration. In an 20% [BMIM][BF4] solution, the DAAO showed 29% activity (compared to the enzyme activity in a buffer), whereas when the [BMIM][BF4] concentration was increased to 60%, the enzyme activity dropped to 15%. For the experiment to generate the data in Table 32 the concentration of [BMIM][BF4] and all the other tested ionic liquids was 50%. According to Lutz-Wahl *et al* [163], the native DAAO showed very high activity in [MMIM][$MMPO_4$], which is also a water miscible IL (129% in 40% solution), but when its concentration was increased to 60% the enzyme activity sharply dropped to 2%.

The enzyme was also very active in a two-phase system made of a buffer and [1-methoxyethyl-3-methylimidazolium][NTf2], 82% of D-ABA was converted. A moderate activity was shown in [BMIM][PF6] and 1-allyl-3-methlyimidazolium] [NTf2].

3.5.4. Conclusions on Ionic Liquid systems as solvent systems for the CCC-bioreactor

There was some enzymatic conversion in the presence of all of eight ionic liquids. The highest D-ABA conversion ratio was found in trihexyltetradecylphosphonium N-cyano cyanamide, [C6, 6, 6, 14P][N(CN)2] (100% conversion) and [1-methoxyethyl-3-methylimidazolium][NTf2] (82% conversion). The lowest conversion ratio was in the presence of [C6, 6, 6, 14P][NTf2] and [EMIM][BNTf2], 25 and 22%, respectively. [BMIM][BF4] was miscible with water and therefore did not create a two-phase system with the buffer.

ILs are solvents of a relatively high viscosity. In order to use an IL as a solvent system for a CCC separation, it needs to be diluted with an organic solvent, which reduces viscosity [161]. However, organic solvent has been shown to inactivate DAAO (section 3.4). Therefore, despite high D-ABA conversion in the presence of some of them, ionic liquids did not show much promise as a solvent system suitable for the CCC-bioreactor and the project continued with ATPS instead.

3.6. Final conclusions on identifying a solvent system for the CCC-bioreactor

A number of ionic liquid (IL) two-phase systems and the range of tested aqueous organic two-phase systems were found unsatisfactory due to the high enzyme instability in those systems.

The enzyme was found to be considerably more stable in some aqueous two-phase systems (ATPS). Moreover these systems are far more widely described in the literature, making it easier to tailor the phase system to match the criteria of an ideal phase system for the CCC-bioreactor.

Two aqueous two-phase systems were selected as a potential phase system for the CCC-bioreactor: 14/14% (w/w) PEG3350/potassium phosphate and 14/14% (w/w) PEG1000/potassium phosphate, both at pH 7.6. Unfavorable partitioning of the enzyme in the first phase system was overcome by applying the reaction of PEGylation of the enzyme. Although, the enzyme showed slightly better stability and equal activity in 14/14% (w/w) PEG3350/potassium phosphate, the 14/14% (w/w)

PEG1000/ potassium phosphate phase system was chosen as a solvent system for the CCC-bioreactor, as it had the advantage of not requiring a PEGylated enzyme.

4. OPTIMISATION OF THE CCC-BIOREACTOR

4.1. Introduction

In this research project, a CCC-bioreactor was developed to obtain enantiomerically pure L-amino butyric acid (L-ABA) from a racemic mixture of amino butyric acid (DL-ABA), consisting of equal amounts of D- and L- forms. As both enantiomers have the same physical properties, their distribution ratio (D-value) between a mobile and a stationary phase is the same, therefore these two forms cannot be separated by the standard CCC operation mode. To separate these two forms, one of them (the unwanted D-form) needs to be derivatised in order to change its D-value.

If a racemic mixture of amino butyric acid (ABA) is injected onto the CCC-bioreactor column, the enantiomeric form D-ABA is oxidised by the enzyme which is immobilised in the stationary phase. The other form, unreacted L-ABA being the target compound, is eluted unchanged from the coil. The enzyme, D-amino acid oxidase (DAAO), present in the stationary phase, oxidases only D-ABA, which is converted into its keto analogue ketobutyric acid (KBA). Since the D-value of the produced KBA and the unreacted L-ABA are different, the separation of these two compounds is possible. However to obtain pure L-ABA, the D-ABA conversion has to be 100%, otherwise unreacted D-ABA would elute together with L-ABA, thus contaminating it.

In a series of experiments conducted in the CCC-bioreactor, the effect of different parameters (substrate and enzyme concentration, temperature, rotational speed, the mobile phase flow rate, sample loop volume) has been tested. In each experiment only one parameter e.g. the substrate injection amount, was varied, keeping all the other parameters fixed. All experiments were conduced on the J-type centrifuge called “Milli CCC”, designed and made in the Brunel Institute for Bioengineering. The volume of the coil was 45.1ml, 2.7mm bore. In all experiments, the solvent system was 14/14% (w/w) PEG1000/potassium phosphate. The stationary phase was the upper, PEG-rich phase in which the enzyme was dissolved, whereas the mobile phase was the lower, salt rich phase.

4.2.Materials and methods

4.2.1. Materials

Enzyme, D-amino acid oxidase (DAAO), was obtained in a frozen form from Ingenza Ltd, Roslin Centre, Edinburgh, UK. The defrosted liquid was centrifuged for 15min at 10,000 g on a Sigma centrifuge (rotor 9.8cm) to remove cell debris. For the bioreactor experiments the supernatant was taken.

The enzyme substrate, which is DL-amino butyric acid (DL-ABA), was obtained from Sigma-Aldrich (Cat. 162663).

Polyethylene glycol (average molecular weight, M_r=1000) was obtained from Sigma-Aldrich (Cat. P3515). Dipotassium hydrogen orthophosphate (Cat. P5240/53) and potassium dihydrogen orthophosphate (Cat. P4800/53) were obtained from Fisher Scientific.

Aqueous two-phase preparation

For all experiments ATPS 14/14% (w/w) PEG1000/potassium phosphate (280g PEG1000, 58.6g potassium dihydrogen orthophosphate, 221.3g dipotassium hydrogen orthophosphate, and 1440 ml water) was used. Since PEG1000 exists in a solid form at room temperature, it was warmed up to 60°C before using, in order to melt it. Next all components were placed into a 2L glass bottle and shaken vigorously until the salt and PEG had dissolved. ATPS was left overnight to cool to room temperature. It was then shaken again, left to settle and both phases were separated using a separation funnel.

4.2.2. CCC operation

Prior to each experiment, the CCC coil was flushed with at least 500ml of water (over 10 coil volumes).

In order to remove all air bubbles from the coil, the CCC was initially operated in a reverse, “empty”, mode at the lowest 500rpm rotational speed. The coil was flushed with 100ml of upper phase and then filled with 100ml of stationary phase (upper phase with the dissolved enzyme). With the appropriate rotational speed set up, the mobile phase was pumped at 2ml/min flow rate till the coil was equilibrated. Next after setting up the desired flow rate, a sample was injected (DL-ABA dissolved in the

lower phase). The entire process from filling the coil to injection took about 30 min in each case. Fractions eluted from CCC were collected in Eppendorf vials.

4.2.3. Sample analysis

4.2.3.1. Amino butyric acid analysis

In order to determine the ABA conversion ratio in the CCC, every 3 min 3 drops of eluted mobile phase were collected. 50µl of this eluent was derivatised with 150µl mixture of ortho-phthalaldehyde and N-isobutyryl-L-cysteine in 0.4M potassium borate buffer, pH 10. After 90s the samples were diluted to 1ml with water. Derivatised samples then were analysed on HPLC using a C18 column (see section 2.5.1.2b).

4.2.3.2. Ketobutyric acid analysis

If KBA analysis was being performed, 50µl was taken for the ABA analysis, as described above and the rest was incubated for 2 min at 100°C in order to stop the possible enzymatic reaction.

Next 200µl of each fraction was mixed with 400µl 150mM potassium phosphate buffer, pH 8 and 400µl 0.05M phenyl hydrazine aqueous solution, in order to derivatise the KBA present (2.5.1.3). Derivatised samples were analysed on HPLC using a C18 column.

4.2.4. Determination of the D-ABA conversion ratio

Fractions were collected every 3 min starting at a point shortly before the expected elution of the first peak. HPLC analysis of each fraction gave the peak area of both L- and D-ABA. The difference between these two areas (L-D) was expressed as a percentage of the L-ABA peak area to give the percent conversion, since L-D is equivalent to the amount of D-ABA that has been converted as L-ABA is unaffected by the reaction. Table 33 shows an example of a table used to calculate the D-ABA conversion ratio eluted from CCC.

Fraction No	Time [min]	Peak area [mVs]			D-ABA conversion [%]
		L-ABA	D-ABA	L-D	
1	**24**	0	0	0	
2	**27**	42	7	34	**82.7**
3	**30**	153	43	110	**71.9**
4	**33**	709	386	323	**45.6**
5	**36**	1337	929	407	**30.5**
6	**39**	1447	1069	377	**26.1**
7	**42**	977	654	323	**33.1**
8	**45**	412	207	204	**49.6**
9	**48**	154	34	119	**77.6**
10	**51**	46	2	44	**95.1**
11	**54**	17	2	15	**88.5**
12	**57**	0	0	0	
Total		**5293**	**333**	**1958**	**37.0**

Table 33 Analyses of D- and L-ABA to determine the % of D-ABA that converted in CCC

4.3. Results and Discussion

4.3.1. Effect of substrate injection amount on the D-ABA conversion ratio

The first parameter tested was the amount of substrate injected. In seven separate experiments, the substrate amount injected onto the CCC column was varied from 6.2mg to 38.1mg, each in the same injection volume of 1.72ml. All the other parameters were fixed (enzyme concentration in the stationary phase 2%, temperature 20°C, rotational speed 1800rpm, flow rate 1ml/min.)

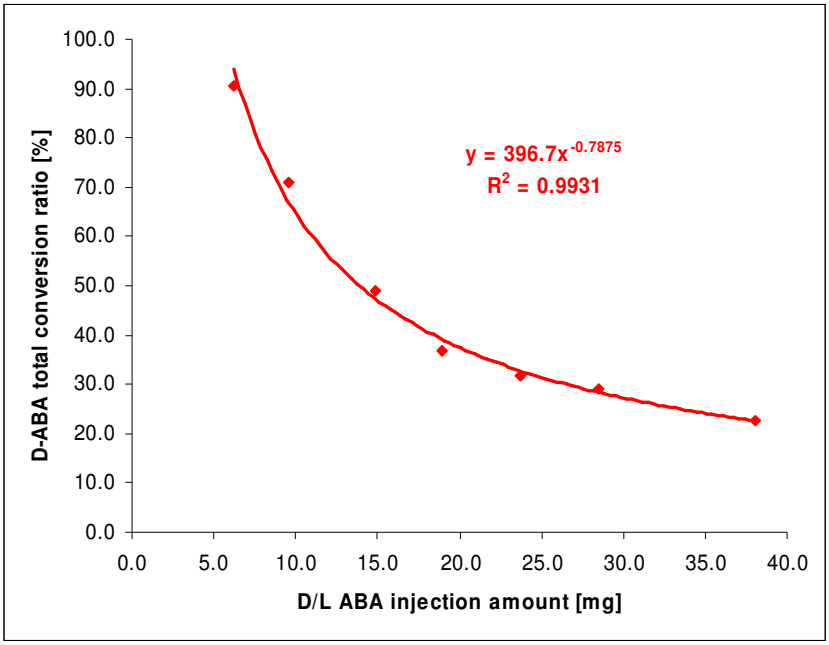


Figure 53 D-ABA total conversion ratio [%] as a function of the amount of DL-ABA injected [mg]. Enzyme concentration in the stationary phase 2%, temperature 20°C, rotational speed 1800rpm, flow rate 1ml/min. The mobile phase was the lower phase of 14/14% (w/w) PEG1000/potassium phosphate pumped from head to tail direction. The stationary phase displaced was in the range of 24-25.5ml.

D-ABA conversion ratio [%] calculated from the HPLC analysis was plotted against the initial DL-ABA injection amount [mg] (Figure 53). The data shows a definite trend with an excellent fit, R^2=0.993. The higher the substrate injection amount, the lower the D-ABA conversion ratio. According to the equation obtained from Figure 53, the substrate injection amount that would be converted with 100% yield is 5.7mg DL ABA, under these experimental conditions.

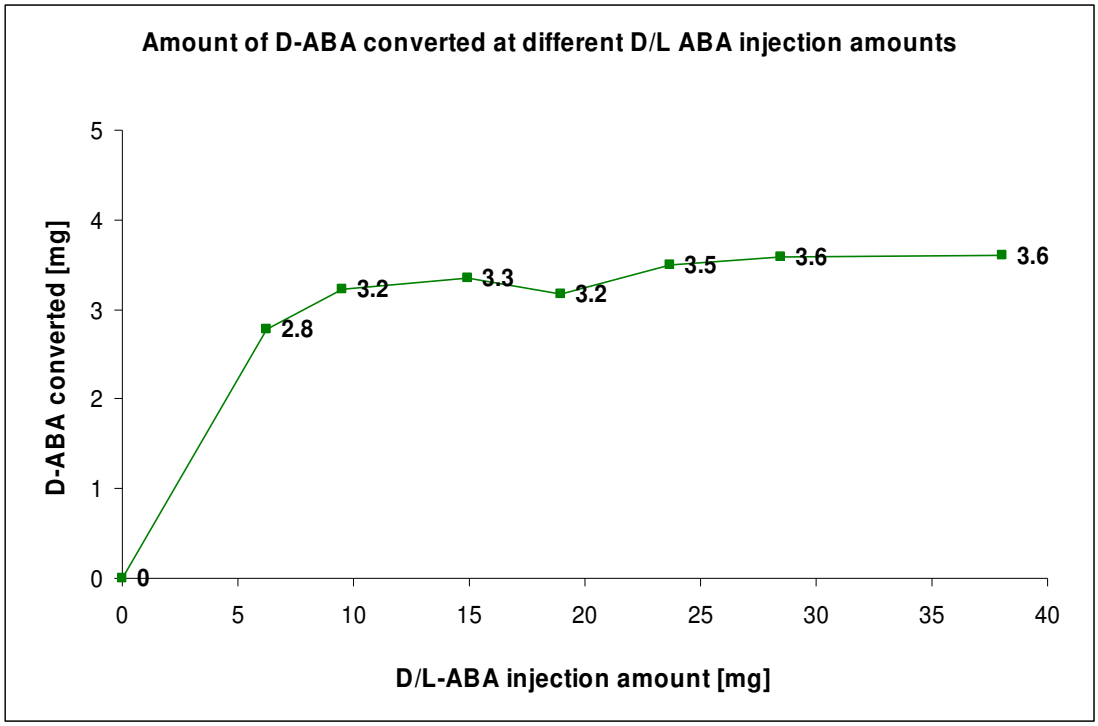


Figure 54 D-ABA converted [mg] against different DL ABA injection amount [mg]

Figure 54 shows the amount of converted D-ABA [mg] was plotted against the amount of injected DL-ABA [mg].

These data can also be analysed by "Lineweaver-Burk" double reciprocal plots to provide an apparent K_m for this system of 3.07mg injected and a maximum conversion of 4.4mg at very high substrate concentration (Figure 55).

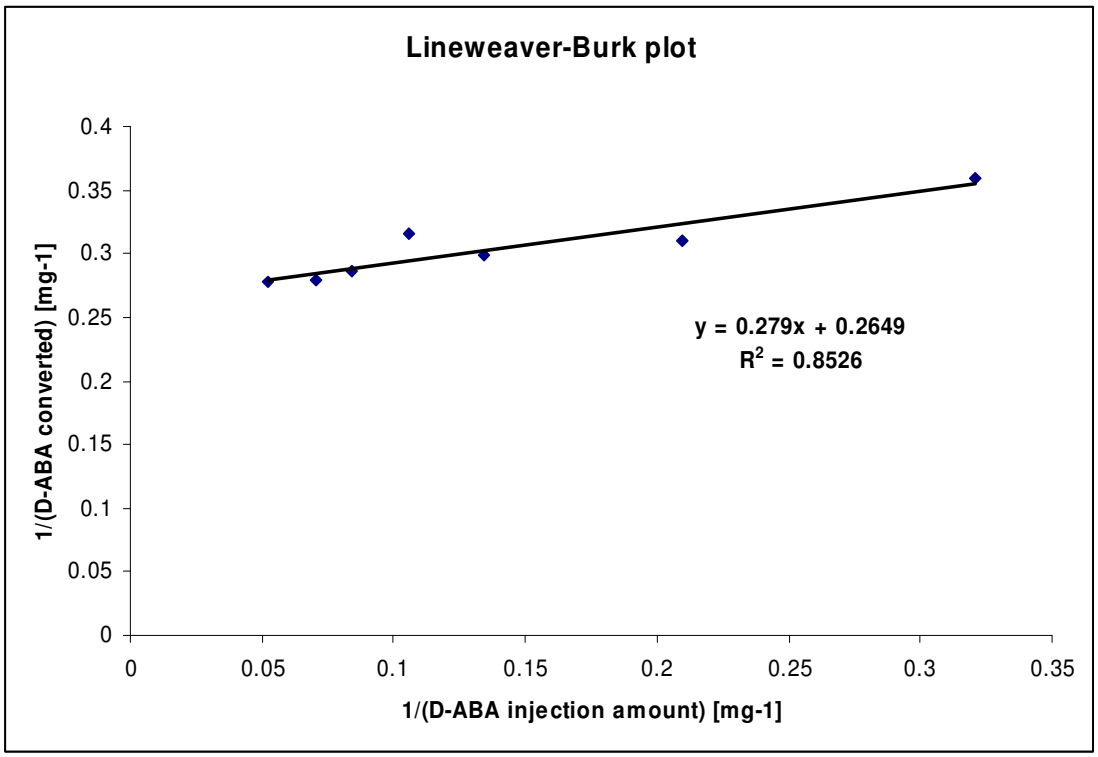


Figure 55 Effect of concentration of DL-ABA on the conversion of D-ABA by DAAO. Data from Figure 54 has been expressed as a double reciprocal plot (Lineweaver-Burk plot) as used in classical enzymology.

These data indicate that the enzyme is behaving with Michealis-Menten kinetics in the CCC-centrifuge and provides evidence for the behaviour of the "bioreactor" feature of the bioreactor-separator, with respect to substrate load, being predictable. This indicates that the bioreaction can be modelled.

4.3.2. Effect of enzyme concentration in the stationary phase on the D-ABA conversion ratio

A series of six experiments was performed in which the only parameter changed was the enzyme supernatant concentration (0.1-4% v/v) in the stationary phase. All the other parameters were constant (substrate injection amount 14.9mg, temperature 20°C, rotational speed 1800rpm, flow rate 1ml/min). In Figure 56, the D-ABA conversion ratio was plotted against the enzyme supernatant % in the stationary phase.

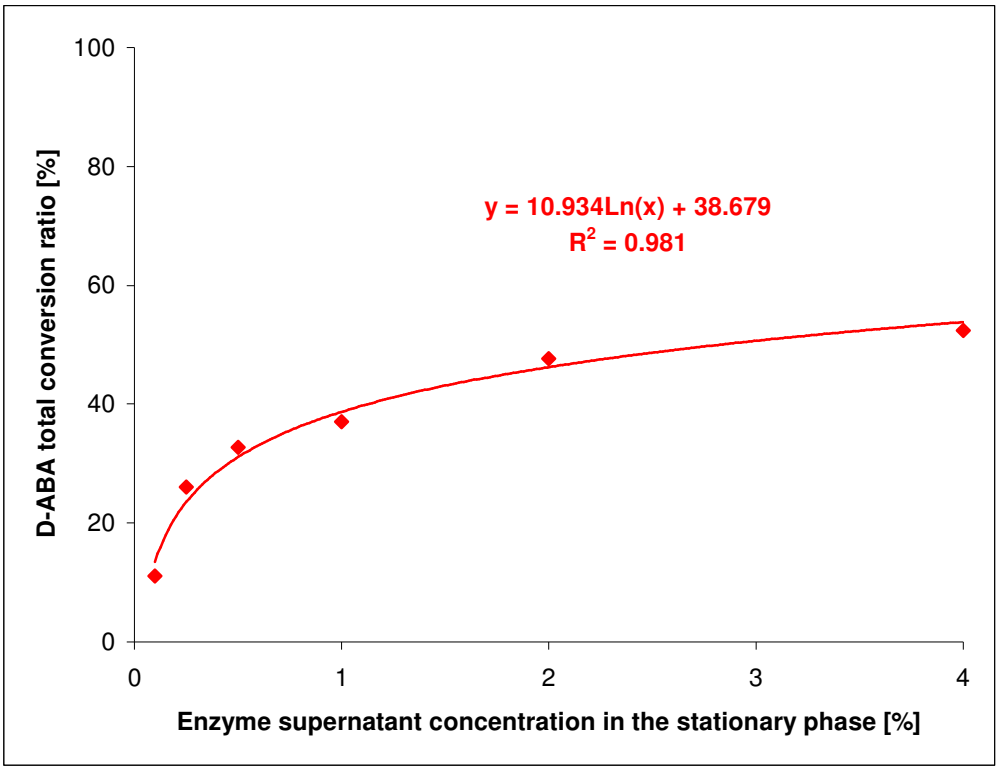


Figure 56 Effect of the enzyme concentration in the stationary phase [%] on the total D-ABA conversion ratio (substrate injection amount 14.9mg, temperature 20°C, rotational speed 1800rpm, flow rate 1ml/min). The mobile phase was the lower phase of 14/14% (w/w) PEG1000/potassium phosphate pumped from head to tail direction. The stationary phase displaced was in the range of 23-27ml.

The shape of the curve is classical for an enzymatic reaction. By increasing the enzyme concentration in the stationary phase, the total D-ABA conversion increases, reaching a plateau at about 2% enzyme in the stationary phase. From that point, even when the enzyme concentration was doubled, the conversion ratio increased only slightly, from 48% (for 2% enzyme) to 52% (for 4% enzyme in the stationary phase). It was therefore concluded that there is no significant advantage in having the enzyme concentration higher than 2% (v/v).

4.3.3. Effect of mobile phase flow rate on the D-ABA conversion ratio

In this experiment, the only parameter changed was the mobile phase flow rate. All other parameters were kept constant (substrate injection amount 14.9mg, enzyme concentration in the stationary phase 2%, temperature 20°C, rotational speed 1800rpm).

According to the chromatography process, a substrate migrates through the CCC coil with the mobile phase. Thus the higher the mobile phase flow rate, the shorter the retention time of a compound. In the case of the CCC-bioreactor, a higher flow rate meant both a shorter time for the substrate being in the column and less contact time with the enzyme.

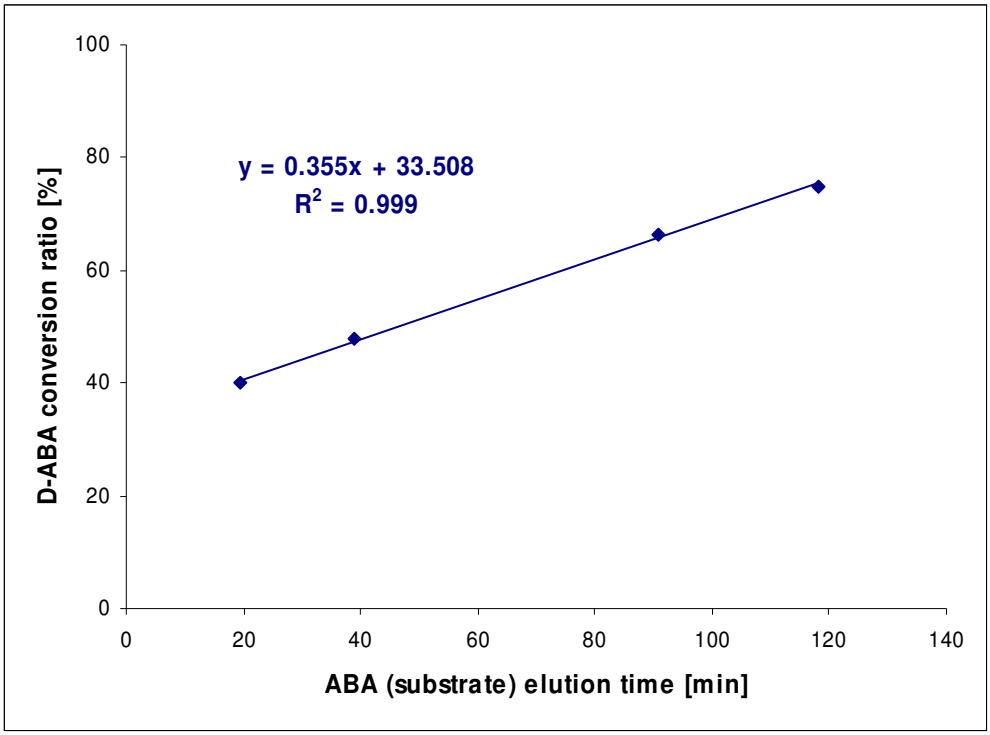


Figure 57 Effect of the mobile phase flow rate on the total D-ABA conversion ratio [%]. In the graph total D-ABA conversion ratio was plotted against the retention time of ABA. The mobile phase was the lower phase of 14/14% (w/w) PEG1000/potassium phosphate pumped from head to tail direction. The stationary phase displaced was in the range of 24-25ml.

As might be expected, it was found that the longer the time of the substrate being in the coil (elution time), the higher the D-ABA conversion ratio (Figure 57). The correlation was linear over this range of flow rates. Extrapolation indicated that 100% conversion for the tested DL-ABA would require 187min, achieved with a flow rate of 0.2 ml/min on this system.

Berthod *et al*[135] have reported that for the catalytic reduction of benzaldehyde to benzylic alcohol by sodium formate in a CCC-bioreactor, more product was obtained with a lower flow rate.

4.3.4. Effect of CCC rotational speed on the D-ABA conversion ratio

Three experiments were conducted at three different rotational speeds (1000, 1400, 1800 rpm) with all the other parameters fixed (substrate injection amount 14.9mg, enzyme concentration in the stationary phase 0.5%, temperature 20°C, flow rate 1ml/min).

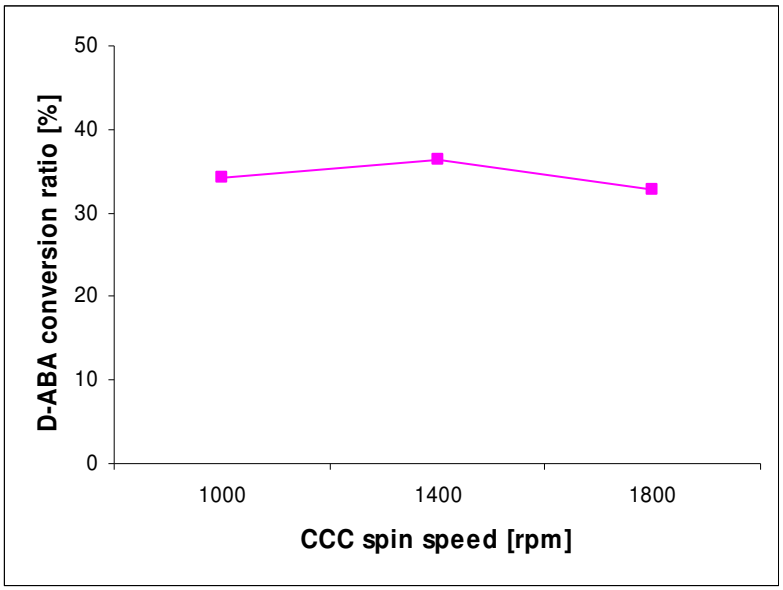


Figure 58 Effect of the CCC rotational speed on the total D-ABA conversion ratio (substrate injection amount 14.9mg, enzyme concentration in the stationary phase 0.5%, temperature 20°C, flow rate 1ml/min). The mobile phase was the lower phase of 14/14% (w/w) PEG1000/potassium phosphate pumped from head to tail direction. The stationary phase displaced was in the range of 23.5-26.5ml.

As shown by the graph, there is no major effect of rotational speed effect on the D-ABA conversion ratio within this rpm range. However it is known[148] that rotational speed has an important effect on stationary phase retention. An increase in rotational speed increases stationary phase retention which will improve the resolution between the unreacted ABA and the KBA product.

4.3.5. Effect of temperature on the D-ABA conversion ratio

In five separate experiments, the only parameter varied was the temperature. Both the CCC coil and the mobile phase reservoir were controlled by a thermostat. The stationary phase was not thermostatically controlled to eliminate the effect of enzyme stability due to temperature before an experiment. It was assumed that after 10 min of

pumping the stationary phase reservoir onto the thermostated coil and a further 15min of coil equilibration using the thermostated mobile phase, the temperature of the stationary phase before injection had reached the target value.

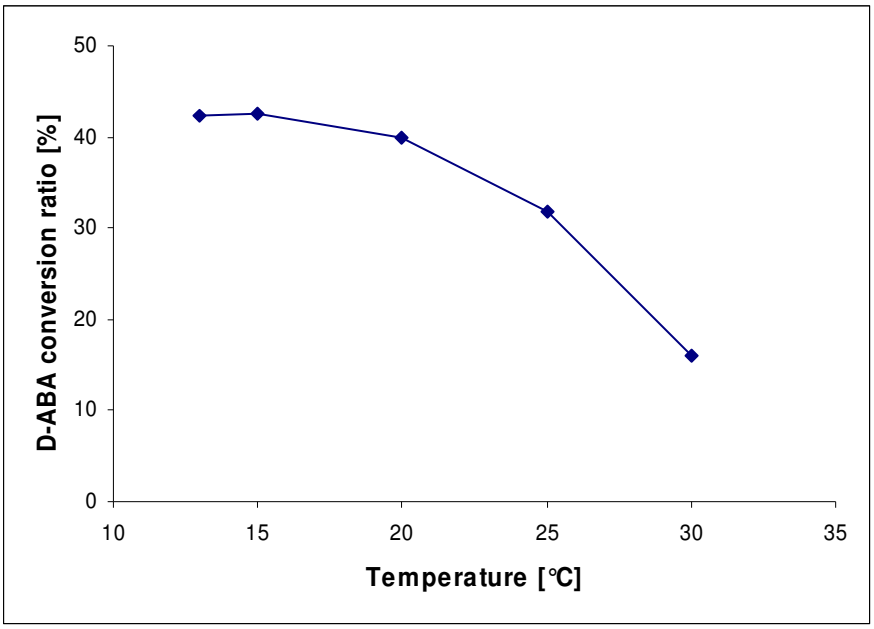


Figure 59 The temperature effect on the D-ABA conversion ratio (substrate injection amount 14.9mg, enzyme concentration in the stationary phase 2%, rotational speed 1800rpm, flow rate 2ml/min). The mobile phase was the lower phase of 14/14% (w/w) PEG1000/potassium phosphate pumped from head to tail direction. The stationary phase displaced was in the range of 24-26ml.

Figure 59 shows that, with increasing temperature above 15°C, there is a decrease in D-ABA conversion. This was unexpected as generally reactions including enzymes are faster with increasing temperature.

Unlike the classical enzymatic reaction, the D-ABA conversion was highest (42%) at lower temperatures 13-15°C. The higher the temperature, the lower the conversion D-ABA ratio (16% at 30°C).

According to Pollegioni *et al*[164], DAAO from *Trigonopsis variabilis* is the most active within the temperature range of 50 to 55°C and is fully stable up to 40°C, then shows a decrease and inactivation at 65°C. However the activity was determined in the presence of a relatively weak buffer (15mM). In these CCC-bioreactor experiments, the concentration of PEG and salt in ATPS is much higher (14g salt and 14g PEG per 100g ATPS), and this may be the cause of the higher activity at lower temperature.

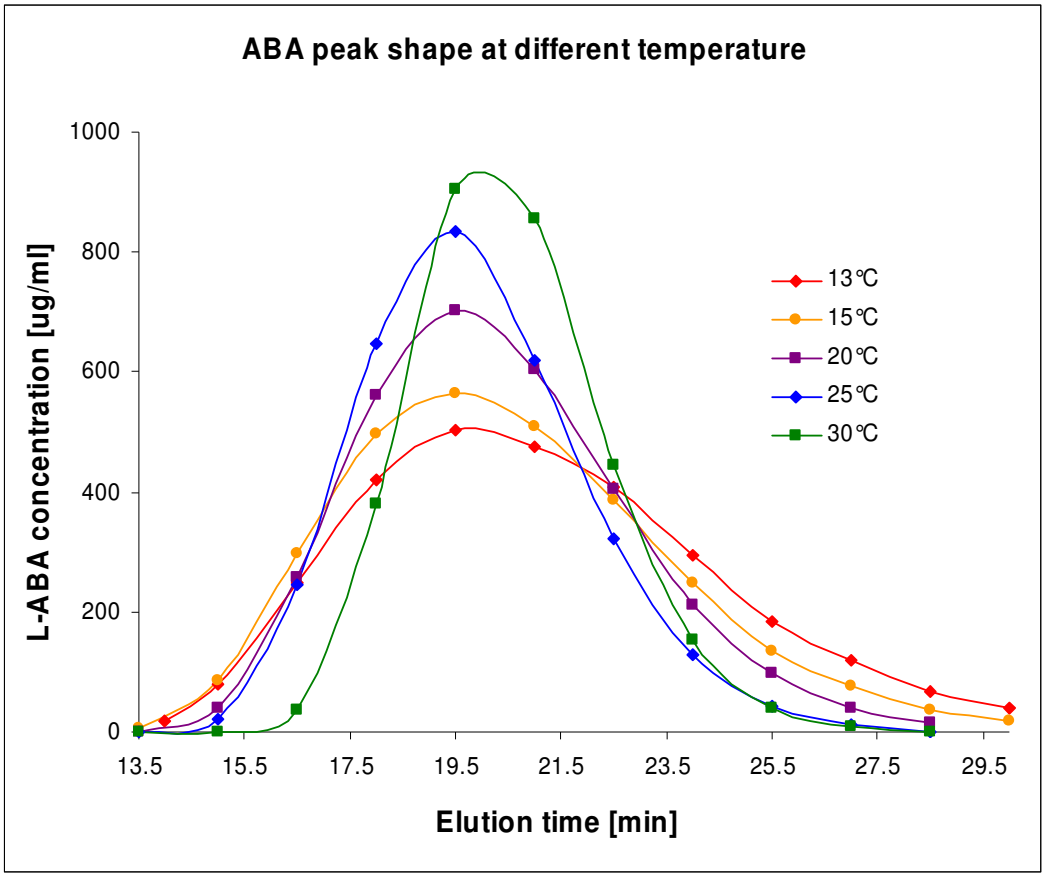


Figure 60 The temperature effect of the ABA peak shape. Substrate injection amount 14.9mg, enzyme concentration in the stationary phase 2%, rotational speed 1800rpm, flow rate 2ml/min. The mobile phase was the lower phase of 14/14% (w/w) PEG1000/potassium phosphate pumped from head to tail direction. The stationary phase displaced was in the range of 24-26ml.

The lower conversion at a higher temperature might also be explained by the temperature effect on the ABA peak shape as it elutes from the CCC-bioreactor. In Figure 60, the HPLC peak area of L-ABA is plotted against elution time from the CCC. Moreover, Table 34 shows the width of each peak [ml] at 10% of a peak height, measured from the baseline. There is a correlation between the temperature and the shape of the peak. The higher the temperature, the narrower the peak. This effect is well known in chromatography. It is caused by a lower viscosity of liquid phases at a higher temperature, which gives better mixing and thus higher efficiency.

Temperature [°C]	Peak width [min]
13	15.7
15	13.3
20	11.0
22	9.1
30	7.7

Table 34 Peak width [min] measured at 10% of a peak height from the baseline. Data measured from peaks shown in Figure 60.

Although the sharper peak is good for the separation process, it might not be desired for the bioconversion. The substrate migrates through the coil with the mobile phase as a single bolus. At any moment, the only enzyme working is that in contact with the small bolus of substrate. At 30°C a narrow and concentrated slug of the substrate passes through the coil, involving a small fraction of the enzyme within the whole length of the coil at any one time. The rest of the enzyme is idle. Thus at 30°C a very small amount of the enzyme struggles with the relatively concentrated bolus of the substrate. In contrast, decreasing the temperature to 15°C, the resolution of the ABA is poorer. The substrate passes through the coil as a broader, less concentrated slug, involving more enzyme. It is possibly that this effect might have caused the higher D-ABA conversion at lower temperatures.

4.3.6. Effect of injection volume on the D-ABA conversion ratio

Four experiments were performed to determine the effect of substrate concentration on the conversion ratio. Using four different sample loops, the same amount of substrate at different concentrations was injected onto the coil.

Sample loop volume [ml]	% of the 45.1ml CCC coil	D/L-ABA injection amount [mg]	D/L ABA concentration [mg/ml]	D-ABA conversion ratio [%]
0.56	1.2	8.0	14.3	60.4
1.24	2.7	8.0	6.5	60.2
2.48	5.5	8.0	3.2	58.4
4.95	11.0	8.0	1.6	62.7

Table 35 Effect of injection volume on the D-ABA conversion ratio. (substrate injection amount 8mg, enzyme concentration in the stationary phase 2%, temperature 20°C, flow rate 2ml/min, rotational speed 1800rpm).

In the four experiments performed (Table 35), the percentage of D-ABA converted ranged from 58.4-62.7%. No trend between the conversion and the sample loop volume was observed. The small difference in conversion is assumed to be within experimental error. It was expected that the D-ABA conversion would increase when a bigger sample loop and a less concentrated sample was used, keeping the amount of sample injected the same. It was thought that it would be easier to convert a less concentrated substrate passing through the column rather than a very concentrated small slug of it.

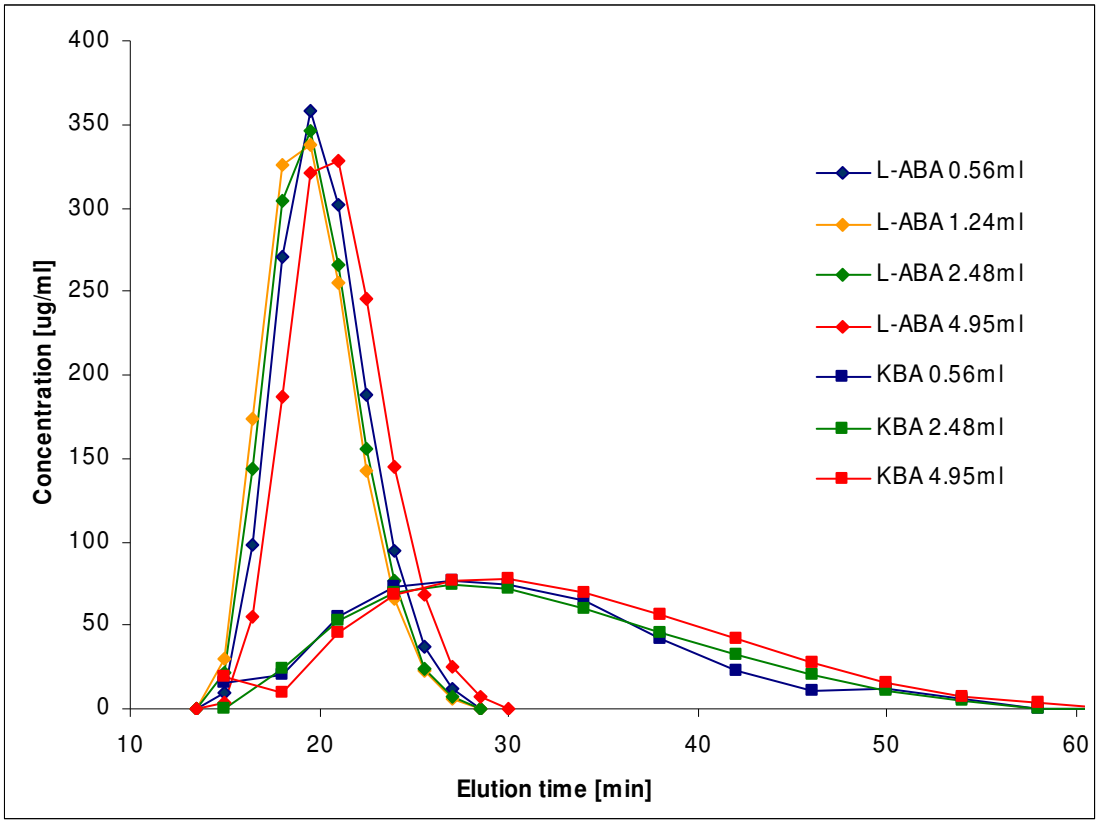


Figure 61 L-ABA (first peak) and KBA (second peak) in fractions collected from CCC. Four experiments were performed each time using different volumes of sample loop (marked by different colours). Substrate injection amount 8mg, enzyme concentration in the stationary phase 2%, temperature 20°C, flow rate 2ml/min, rotational speed 1800rpm. The mobile phase was the lower phase of 14/14% (w/w) PEG1000/potassium phosphate pumped from head to tail direction. The stationary phase displaced was in the range of 25-26ml.

There was no significant difference in peak shape and retention time between the peaks obtained from the four experiments (Figure 61). Even when the largest sample loop was used (4.95ml), which is nearly 11% of the column volume, the same resolution was obtained. However, it is known in chromatography that often a better resolution is obtained if a sample is injected in a smaller volume. In the current experiment there was no difference between sample loops from 1.2 % of the CCC coil volume to nearly 11%.

4.3.7. Enzyme degradation within the CCC instrument

The experiment was performed to investigate the effect of physical conditions in the CCC on the enzyme stability. It was suspected that friction between the two phases or the high g-force within the instrument might be contributing to the enzyme inactivation. To investigate this, the enzyme activity in the stationary phase blown out from the CCC was compared with the activity of enzyme simultaneously incubated in the same phase system for the same period of time. In the second case the enzyme was not exposed to the physical conditions within the CCC. The experiment was performed at two temperatures: 20°C and 30°C.

2ml of the enzyme supernatant was dissolved in 98ml of the upper, PEG-rich phase of 14/14% PEG1000/ammonium phosphate (stationary phase). Next the enzyme activity in the stationary phase was determined by colorimetric assay and expressed as the initial activity. 5ml of that phase was taken as a control and incubated at room temperature, measured as 22°C. The rest of the prepared stationary phase was used to fill the CCC column, warmed up to 20°C, the temperature at which the CCC is usually operated. Next the mobile phase was pumped at a flow rate of 2ml/min while the column was rotating at 1800rpm. A proportion of stationary phase was displaced by the mobile phase until equilibrium had been achieved. The enzyme activity was determined in the stationary phase displaced as well as in the control, incubated at room temperature (22°C). The mobile phase was then pumped through the column continuously. After 2h the flow rate was stopped and all the coil content was blown out by compressed air. The enzyme activity was determined for the extruded stationary phase and also for the control (the upper phase incubated at 22°C).

The same experiment was done at 30°C in which the temperature of the CCC coil was 30°C and the enzyme was incubated in the upper phase at 30°C.

Since the two experiments were performed one after the other, for each one the control was the enzymatic activity of aqueous enzyme solutions incubated at room temperature (22°C). Results are presented in Table 36 and Figure 62.

Enzyme activity at 20°C [abs/min]				
	Time [min]	**Control 1 - enzyme activity in water (22°C)**	**Enzyme activity in the upper phase incubated at room temp (22°C)**	**Enzyme activity in the stationary phase from the CCC run at 20°C**
Activity [abs/min]	**0**	0.232	0.198	0.198
	50	0.196	0.131	0.113
	120	0.210	0.121	0.033
St. error	**0**	0.006	0.004	0.004
	50	0.001	0.009	0.003
	120	0.003	0.002	0.001

Enzyme activity at 30°C [abs/min]				
	Time [min]	**Control 2 - enzyme activity in water (22°C)**	**Enzyme activity in the upper phase incubated at 30°C**	**Enzyme activity in the stationary phase from the CCC run at 30°C**
Activity [abs/min]	**0**	0.224	0.211	0.211
	55	0.186	0.091	0.064
	120	0.198	0.052	0.002
St. error	**0**	0.002	0.002	0.002
	55	0.001	0.003	0.001
	120	0.003	0.002	0.000

Table 36 Enzyme stability in the phase system from CCC by the colorimetric assay.

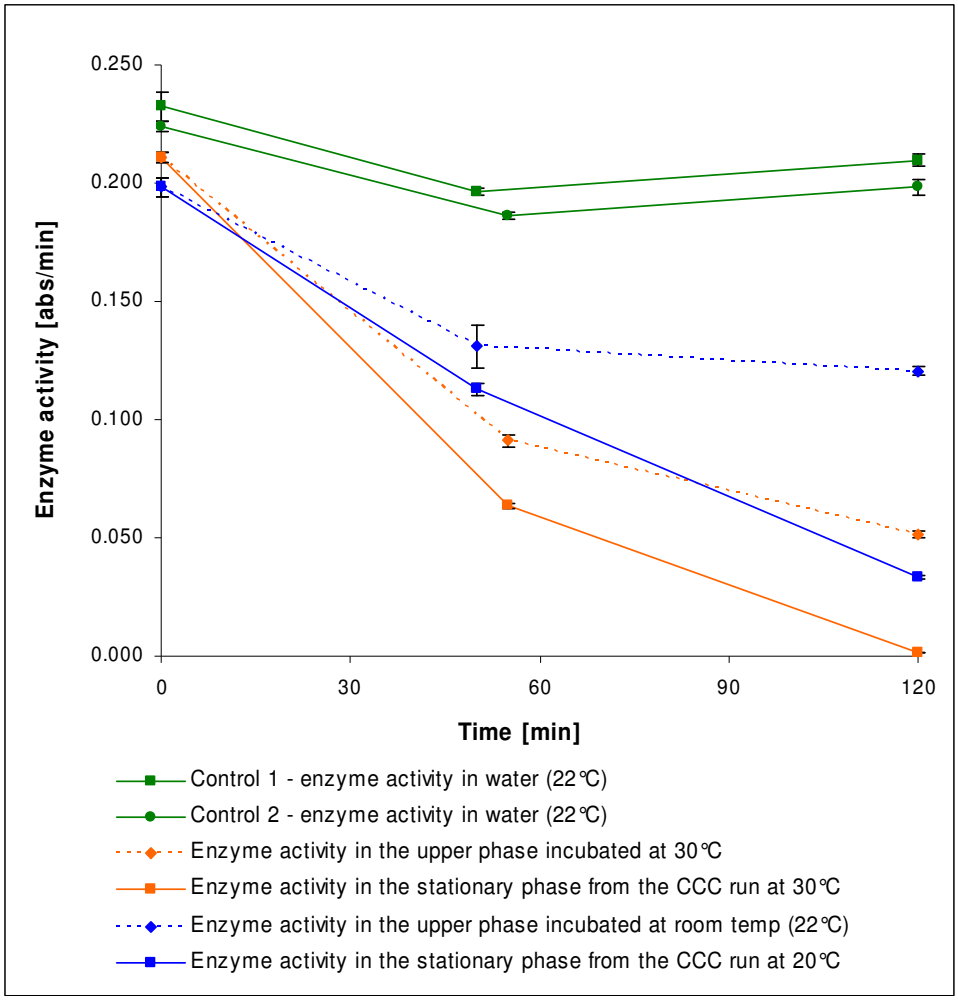


Figure 62 Enzyme stability (by the colorimetric assay) in the stationary phase of the CCC, operated at 20°C and 30°C.

The activity of the enzyme in the stationary phase blown out from the CCC was lower than that of the control (incubated upper phase with the enzyme), at both the tested temperatures. There was hardly any activity (1%) in the stationary phase after 2h when the CCC was operated at 30°C and about 17% of the initial activity when the CCC was operated at 20°C. Thus at the higher temperature (30°C) the enzyme seems to lose activity quicker both in the CCC as well as in the control.

The conclusion that can be drawn from this experiment is that the CCC should be operated at 20°C rather than 30°C. The D-value of the enzyme in 14/14% PEG1000/ammonium phosphate ATPS determined at room temperature was 19. Therefore, it is not known if the lower activity in the stationary phase blown from the column equilibrated at 30°C was caused by the enzyme activity declining or its unintentional elution with the mobile phase during the CCC run as the higher temperature might have affected the partitioning of the enzyme. That can be determined by measuring enzyme protein concentration in the stationary phase.

Thus in the following experiment two assays were performed. The colorimetric enzyme assay was used to determine the enzyme activity and Bradford assay to measure enzyme protein present in the stationary phase. It had previously been demonstrated that the upper phase of 14/14% PEG1000/ammonium phosphate does not interfere with the Bradford assay (2.5.4.2).

The stationary phase was made by dissolving 2ml of enzyme supernatant in 98ml of the upper phase of 14/14% PEG1000/ammonium phosphate. Then both the initial enzyme activity (colorimetric assay) and the enzyme concentration (Bradford assay, section 2.5.4) were determined. After that, 2ml of the stationary phase was mixed with 2ml of the lower phase and placed on the rotary wheel (room temperature). This was a control for the CCC. The rest of the stationary phase was pumped onto the column warmed up to 20°C. Next the mobile phase was pumped at a flow rate of 1ml/min while the coil was rotating at 1800rpm. After 1.5h the flow rate and the coil rotation were stopped and the stationary phase blown out by compressed air. The enzyme concentration and activity was determined again (Final). The activity in the phase system on the wheel (control) was also determined in the upper phase after the phases had settled down. In Table 37 it can be seen that in the stationary phase, blown out from the coil, both the enzyme concentration and its activity are lower than the initial

values. There was 76% of the initial enzyme concentration detected and only 27% of the initial activity in the stationary phase blown out from the coil, which shows a greater loss of the activity than enzyme concentration.

	Enzyme activity [abs/min]				Enzyme concentration [mg/ml]	SD
	Initial	SD	Final (2h)	SD		
Enzyme in water	0.213	0.009	0.231	0.003	0.42	0.043
Upper phase from the wheel	0.214	0.001	0.138	0.005	0.46	0.017
Upper phase from the CCC	0.214	0.001	0.063	0.002	0.35	0.017

Table 37 DAAO activity (colorimetric enzyme assay) and protein enzyme concentration (Bradford method) determined in the stationary phase. The enzyme concentration was determined at the final 2 hour point. The measurement was performed in triplicate.

Figure 63 shows the values for specific activity of the enzyme calculated as the ratio of enzyme activity to the protein concentration. This ratio, just after the enzyme was added to the upper phase, was 0.47 and dropped to 0.30 for the stationary phase incubated on the wheel for 2h (control). In the case of the phase blown out from the CCC column, the loss of enzyme activity was even bigger and the final ratio was 0.18. It shows that not only the ATPS but also the CCC increases the enzyme instability.

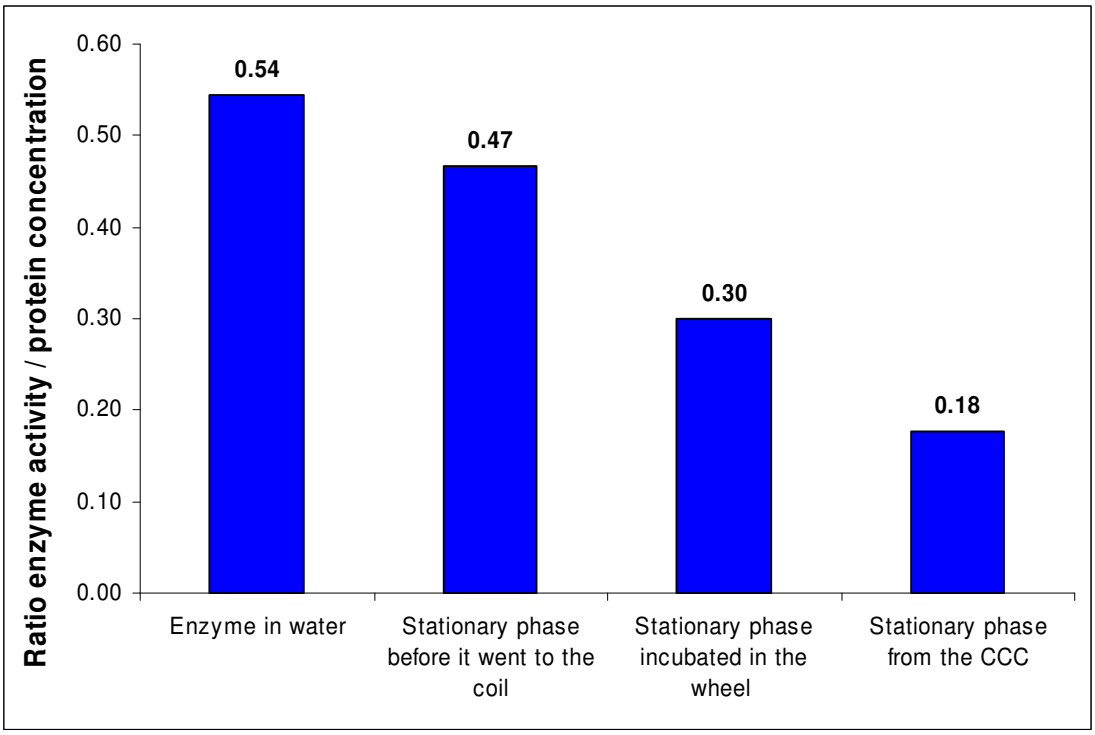


Figure 63 Ratio of the enzyme activity to the enzyme protein concentration for water (control), stationary phase before pumping onto the CCC column, stationary phase from the CCC, stationary phase (upper phase) from the wheel.

4.3.8. Effect of oxygen addition on the D-ABA conversion ratio

An important parameter, which may limit the D-ABA conversion, may be the oxygen content. DAAO, as a flavoenzyme catalyst, conducts an O_2-dependent transformation of amino acids. Since the reaction takes places inside the CCC coil, the whole available oxygen must be dissolved in the solvent. This is made even more difficult as the salt concentration in the mobile phase is over 30% and this reduces the content of dissolved oxygen.

Three experiments were performed using ATPS 14/14% (w/w) PEG1000/ potassium phosphate (the substrate injection amount 14.9mg, 2% enzyme in the stationary phase, rotational speed 1800rpm, flow rate 2ml/min, CCC temperature 20°C). The mobile phase temperature was 6°C. The mobile phase was oxygenated by bubbling pure oxygen into it with a nozzle. Two control experiments were performed. In both cases the mobile phase (6°C and 20°C) was not oxygenated.

Temperature of CCC/temperature of MP	D-ABA conversion [%]
20℃ / 20℃	40
20℃ / 6℃	41.6
20℃ / 6℃ oxygenated	46.5

Table 38 D-ABA conversion ratio [%] obtained when the oxygenated mobile phase (MP) at 6°C was used. For the control, two experiments with non-oxygenated mobile phase at 6°C and 20°C were performed.

It is well known that the oxygen solubility increases as the temperature decreases. In the current experiment, decreasing the temperature from 20° to 6° did not improve the reaction yield by much. By saturating the mobile phase with oxygen, its concentration in solution was estimated to increase 5 fold. However the D-ABA conversion increased only 4% compared to the control. In conclusion, there is no major benefit from bubbling the mobile phase with pure oxygen.

4.3.9. Effect of catalase addition on the D-ABA conversion ratio

The catalytic activity of DAAO on D-ABA leads to KBA, ammonia and H_2O_2 (hydrogen peroxide), which is a by-product of the reaction. H_2O_2 is a strongly oxidising agent, therefore it might inactivate the enzyme by oxidation of some sensitive residues of the protein (methionine, cysteine). Pollegioni *et al*[110] reported partial inactivation of DAAO in the presence of hydrogen peroxide but at a high concentration of H_2O_2 (100 mM). The most sensitive enzyme to the presence of hydrogen peroxide was DAAO isolated from pork kidney, which after 2 hours maintained only 28% of the initial activity. DAAO from *Rhodotorula gracilia* and *Trigonopsis variabilis* was more stable, maintaining, respectively 60, and 80% of the initial activity after 2 hours.

Catalase, which can be found in nearly all organisms exposed to oxygen, catalyses the decomposition of hydrogen peroxide to oxygen and water. It has been found that co-immobilization of DAAO with catalase eliminates much more hydrogen peroxide in comparison to the separate immobilization of the two enzymes[165].

Since catalase degrades H_2O_2, releasing free oxygen which is required for D-ABA conversion, the presence of catalase in the CCC-bioreactor on the yield of the reaction was investigated. Five experiments were performed, varying the amount of catalase

added to the stationary phase, while the D-ABA conversion ratio was recorded. Results are presented in Figure 64.

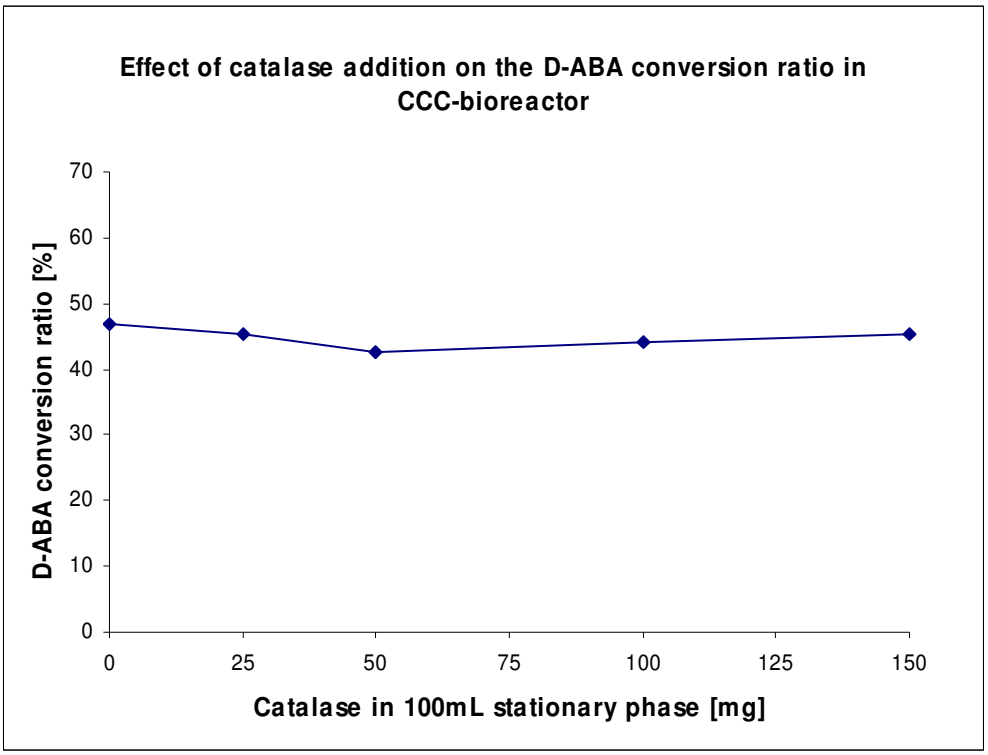


Figure 64 Effect of catalase present in the stationary phase on the D-ABA conversion ratio. The concentration of catalase was varied from 0 to 150mg in 100ml of the stationary phase. All other parameters were fixed (substrate injection amount 14.8mg, enzyme concentration in the stationary phase 2%, rotational speed 1800rpm, flow rate 1ml/min, temperature 22°). The mobile phase was the lower phase of 14/14% (w/w) PEG1000/potassium phosphate pumped from head to tail direction. The stationary phase displaced was in the range of 23-24ml.

The conclusion was that the addition of catalase to the stationary phase did not seem to affect the D-ABA conversion ratio (Figure 64). The achieved D-ABA conversion ratio was between 44 and 46.9% with the highest of these values at 0ml catalase added, therefore no advantage was seen with the use of catalase and the CCC-bioreactor was subsequently operated without its addition.

4.3.10. Repetitive substrate injection

It was clearly a big advantage to the technique if repetitive injections were possible. That would allow use of the same phase system and enzyme several times. To test this possibility, equal amounts of substrate were injected 4 times into the

column every 20 min. Fractions of the mobile phase coming from CCC were analysed for ABA to determine the total D-ABA conversion ratio for each CCC injection. The results are presented in Figure 65.

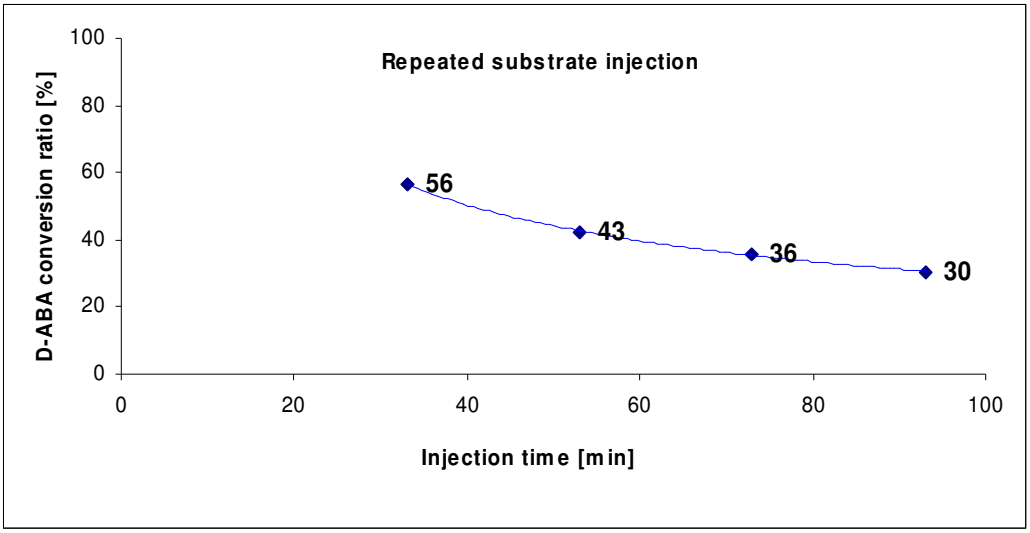


Figure 65 Conversion ratio obtained for the repetitive DL-ABA injection. The same amount of DL-ABA (9.5mg) was injected every 20 min. (enzyme concentration in the stationary phase 2%, rotational speed 1800rpm, flow rate 2ml/min, temperature 20°C). The time was started when the enzyme was added to the stationary phase.

The D-ABA conversion ratio was highest for the first injection (56%). Then the conversion ratio gradually decreased, reaching 30% for the last injection.

The target, which is pure L-ABA, can only be achieved if 100% D-ABA is converted within the CCC. From Figure 65, the conversion achieved for each injection was lower than the previous injection. A 100% conversion may be possible for the first injection by optimising the run parameters but seems unlikely to be achieved for the second injection. Therefore, only one injection is possible. Even a small amount of unconverted D-ABA appearing during next injection would contaminate the product.

4.4. Conclusion

Experiments presented in this chapter established the effect of various parameters such as substrate and enzyme concentration, temperature, rotational speed, the mobile phase flow rate, sample loop volume, mobile phase oxygenation and the presence of catalase, on the D-ABA conversion ratio in the CCC-bioreactor.

The bioconversion of D-ABA depends most on the substrate injection amount and the mobile phase flow rate. In order to achieve a high conversion, a relatively small amount of the substrate at a low mobile phase flow rate needs to be injected. Other important parameters are the concentration of enzyme in the stationary phase and the temperature of the coil. The enzyme protein concentration at the received activity in the stationary phase should be around 0.4mg/ml. The optimal temperature is 20°C. The bioconversion of D-ABA was not dependent on the rotational speed of the CCC coil. Neither the presence of catalase nor the sample loop volume had any effect on the analysed process within the parameter ranges tested.

Table 39 shows the optimal conditions of the CCC-bioreactor which are likely to produce 100% D-ABA conversion.

Parameter	**Value**
Substrate injection amount	8.0mg
Enzyme concentration	2% [v/v]
Mobile phase flow rate	0.5ml/min
CCC rotational speed	1800rpm
Temperature	20°C
Injection volume	1.72ml
Oxygen addition	None
Catalase addition	None
Injection repeats	No repeats

Table 39 Operational conditions of the CCC-bioreactor to achieve 100% conversion of D-ABA.

5. BIOCONVERSION AND SEPARATION IN COUNTERCURRENT CHROMATOGRAPHY

5.1. Introduction

In this chapter, the successfully performed CCC-bioreactor is described (5.3.3). By selecting the solvent system indicated in chapter 3 and the operating conditions optimised in Chapter 4, L-ABA of 99% enantiomeric purity was achieved in a single step totally separated from KBA. To achieve a total L-ABA and KBA separation, a Multiple Dual-Mode was applied.

The CCC can be operated in two modes, either in Single Flow Mode (5.2) or Multiple Dual-Mode (5.3). Single Flow Mode is the standard mode, when the direction of the mobile phase is the same during the whole separation. In this case, the mobile phase was the lower salt-rich phase pumped from the head to the tail of the column.

Since both the mobile phase as well as the stationary phase are liquids, the flow direction and the pumped phase can be altered at any time so that the mobile phase becomes the stationary and *vice versa*. This is called the Multiple Dual-Mode (MDM)[166].

5.2. Bioconversion and separation using single flow mode

In this mode the column was initially filled with a stationary, upper phase where the enzyme was partitioned and the lower phase was pumped as the mobile phase. These experiments were performed on Milli CCC (2.2.1). The coil was rotated at 1800rpm in a clockwise direction. The mobile, salt-rich phase was pumped from head to tail direction at a flow rate of 0.5ml/min.

5.2.1. Separation of ABA and KBA in CCC

Before performing the CCC-bioreactor, the resolution of ABA and KBA was tested in the system. The upper, PEG-rich phase was used as the stationary phase. There was no enzyme in the stationary phase as only a separation was performed.

To predict the elution time and a shape of KBA, the theoretical model design by Joost de Folter and Ian Sutherland [167] was used. The program can predict an elution time and shape of a peak based on a D-value of a compound, the mobile phase flow rate, stationary phase retention and a number of mixing and settling zones during a separation. For the D-values of ABA and KBA, a clear separation was predicted. Results are presented in Figure 66.

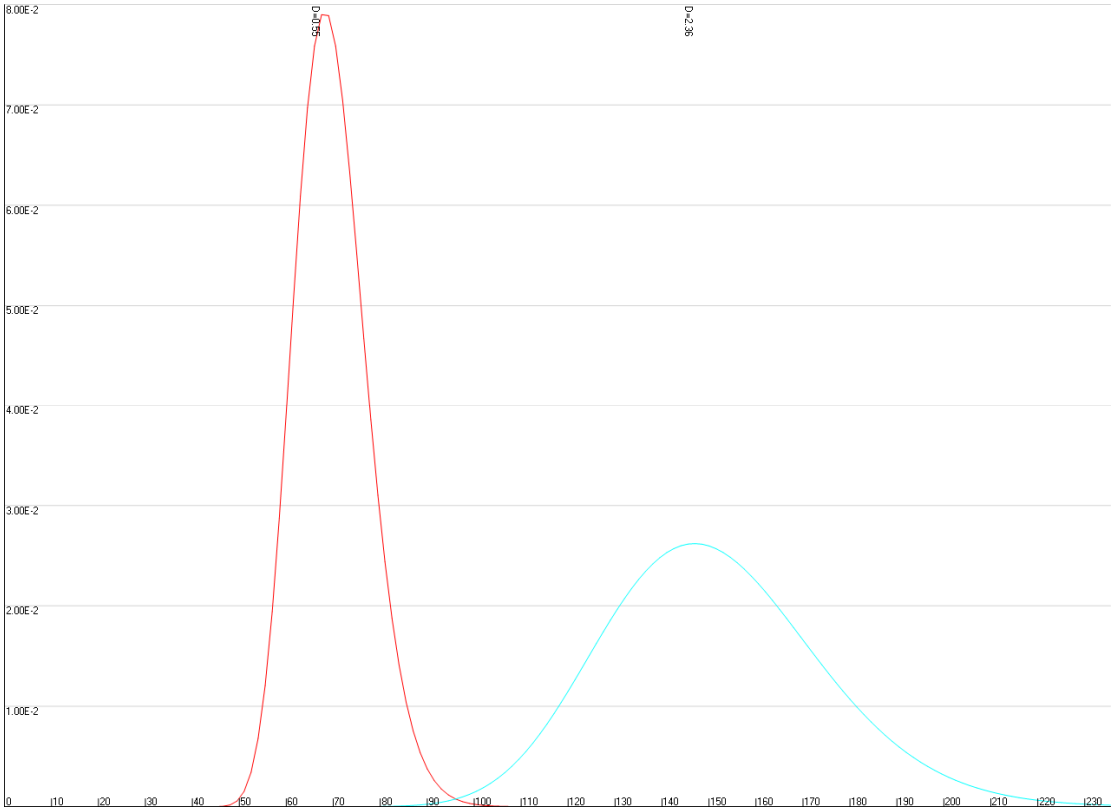


Figure 66 Theoretical model generated by the program CCC2 made by Joost de Folter and Ian Sutherland at Brunel Institute for Bioengineering, Brunel University. The program can be used to predict the shape and elution time of peaks on the basis of their D-value, as well as the mobile phase flow rate and stationary phase retention.

This was confirmed experimentally. A mixture of DL-ABA (8.1mg) and KBA (4.4mg) was injected onto the equilibrated column. Fractions of the mobile phase eluting from CCC were collected every 3min and analysed for ABA and KBA. A reconstructed chromatogram, based on the HPLC analysis, is presented in Figure 67 which agrees with the theoretical profile of Figure 66.

The two injected components were almost completely separated. The resolution factor is 1.03. There is a strong similarity between the chromatogram from the obtained experimental data (Figure 67) and the chromatogram from the theoretical model (Figure 66).

Since the separation of ABA and KBA at the chosen conditions was possible, the next step would be performing the CCC-bioreactor with the enzyme present in the stationary phase, so the separation would become that of the unreacted L-ABA with KBA formed within the coil.

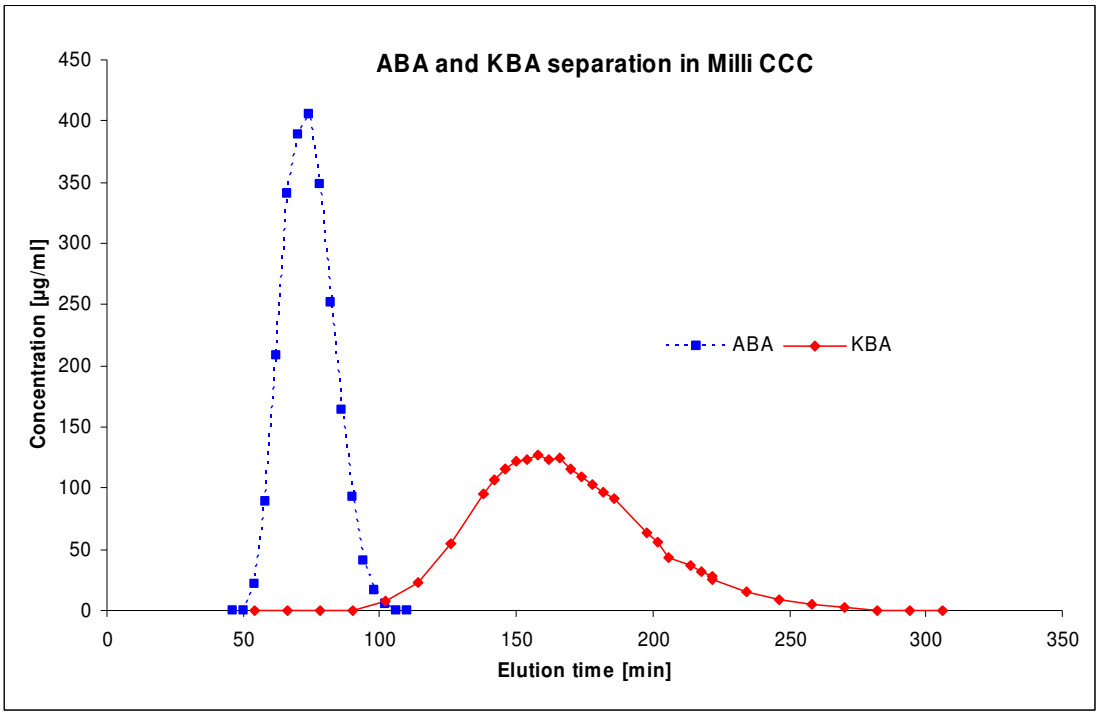


Figure 67 Resolution of ABA and KBA in the Milli CCC. The phase system used was 14/14% (w/w) PEG1000/potassium phosphate. The PEG-rich phase was used as the stationary phase. The flow rate of the mobile, salt-rich, phase pumped in the head to tail direction, was 0.5ml/min. The coil (45ml) was rotated clockwise at 1800rpm. The temperature was 20°C. The stationary phase displaced was 24ml.

5.2.2. Performing the CCC bioreactor-separator

As mentioned before, in order to get a satisfactory L-ABA separation from its racemic mixture, 100% conversion of D-ABA has to be achieved. Otherwise unreacted D-ABA co-eluting with L-ABA would contaminate the product.

Therefore the best conditions (Table 39) for achieving 100% D-ABA conversion were selected based on studies presented in Chapter 4. Thus a relatively small amount of DL-ABA (8mg) was injected onto the CCC. In order to extend the reaction time, the

conversion was performed at a low flow rate (0.5 ml/min). The solvent system was ATPS 14/14% (w/w) PEG1000/potassium phosphate. No extra oxygen or catalase was used. 2ml of the enzyme, DAAO, was mixed with 98ml of the upper phase. The experiment was conducted at 20°C at 1800 rpm rotational speed. Fractions taken during the experiment were analysed for both ABA and KBA. Results are shown in Figure 68 and Figure 69.

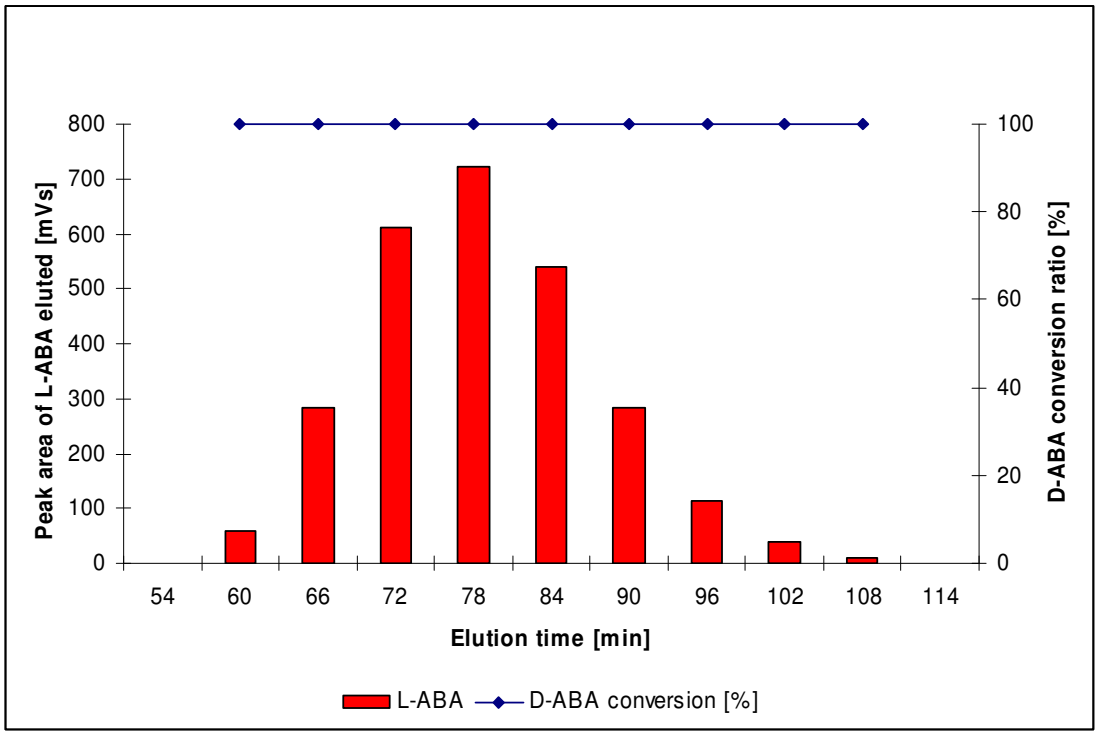


Figure 68 Peak area of L-ABA for fractions taken from the CCC. The dark blue line shows the D-ABA conversion for each fraction.

The retention time for ABA was 78min. Within that time, all injected D-ABA was converted into KBA. Every fraction consisted of only the L-form of ABA (Figure 68 and Figure 69). The dark blue line in Figure 68 shows that 100% conversion of D-ABA was achieved for all fractions.

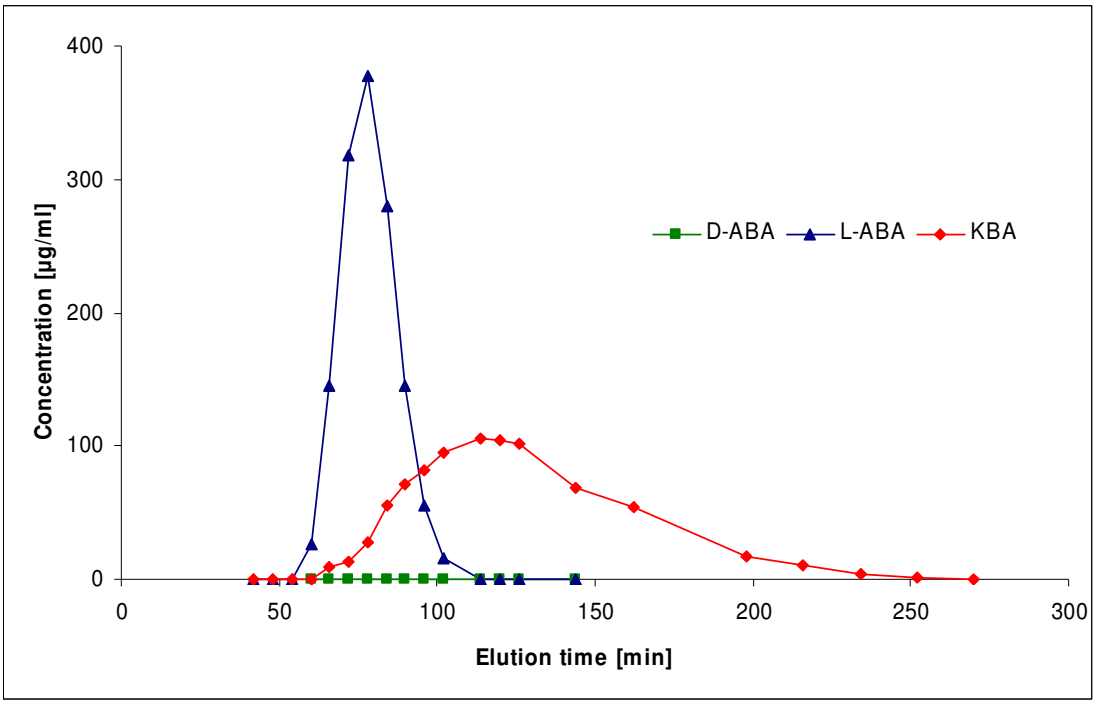


Figure 69 Bioconversion and separation in CCC - single flow mode. Chromatogram reconstructed based on HPLC analysed fractions eluted from CCC. Unreacted L-ABA (blue line) and produced KBA (red line) eluted from the CCC after the bioconversion. 2ml of the enzyme supernatant was mixed with 98ml of the upper, stationary phase of 14/14% (w/w) PEG1000/potassium phosphate. Substrate injection amount 8mg, temperature of the coil 20°C, rotational speed 1800rpm. The mobile phase was the lower phase pumped from head to tail direction at the flow rate of 0.5ml/min. The stationary phase displaced was 25ml. Resolution factor between the peaks was 0.35.

From the CCC, two peaks eluted, unreacted L-ABA (blue line) and produced KBA (red line, Figure 69). L-ABA eluted after 78min, whereas the retention time for produced KBA was 114-120 min. As mentioned before, all the D-ABA was converted to KBA. Thus there was no D-ABA in any fractions. There is a visible separation of unreacted L-ABA and produced KBA. However each fraction of L-ABA was contaminated with a certain amount of produced KBA.

In Figure 69 the retention time of KBA is about 120min. That is different from the time predicted (145min) using the CCC2 program (Figure 66) and confirmed experimentally (Figure 67). It needs to be noted that, as shown in Figure 67, the KBA was injected on the column and passed its whole length before eluting, whereas in Figure 69 DL-ABA was injected and KBA produced inside the column. Since D-ABA was gradually converted as it was travelling within the coil, KBA was produced in various parts of the coil. The KBA produced in a further part of the coil had a

smaller distance to travel and therefore eluted earlier than expected from the CCC2 program.

Moreover, in Figure 69, KBA shows a tailing effect. That can also be explained by the fact that KBA was produced in different parts of the CCC coil, therefore the peak is not symmetric.

Figure 68 and Figure 69 demonstrate that CCC can be used as a simultaneous bioreactor and separator. It was possible to partially isolate the desired L-ABA from the racemic mixture using the CCC-bioreactor. As a bioreactor, it can be optimised towards a high D-ABA conversion ratio by selecting optimal operational parameters. The most critical parameters are the substrate injection amount and the mobile phase flow rate, which determines the reaction time. As demonstrated, it is possible to achieve 100% conversion. However the separation needs to be improved.

5.2.3. Centrifugal Partition Chromatography (CPC)

Despite 100% D-ABA conversion being achieved, the separation in CCC was not satisfactory as L-ABA coeluted with KBA (Figure 69). Since the cascade phase mixing in CPC is far more efficient than the wave mixing that is characteristic for CCC, the CPC (1.3.4.2) was examined as a bioreactor-separator for the comparative purposes. The following experiments were performed on a CPC centrifuge purchased from Armen Instrument (2.3).

5.2.3.1. Separation using CPC

Before performing the CPC-bioreactor, the ability of CPC to separate of ABA and KBA was tested. There was no enzyme in the stationary phase as the CPC was used only as a separator. The phase system used was 14/14% PEG1000/potassium phosphate. The stationary phase was the upper, PEG-rich phase. The coil was rotated at 2000rpm. The mobile, salt-rich phase was pumped from direction at flow rate 5ml/min in descending mode.

On the equilibrated column, a mixture of ABA and KBA dissolved in the lower phase was injected. Fractions were collected every 5 min and analysed on HPLC for ABA and KBA. The reconstructed chromatogram is presented in Figure 70.

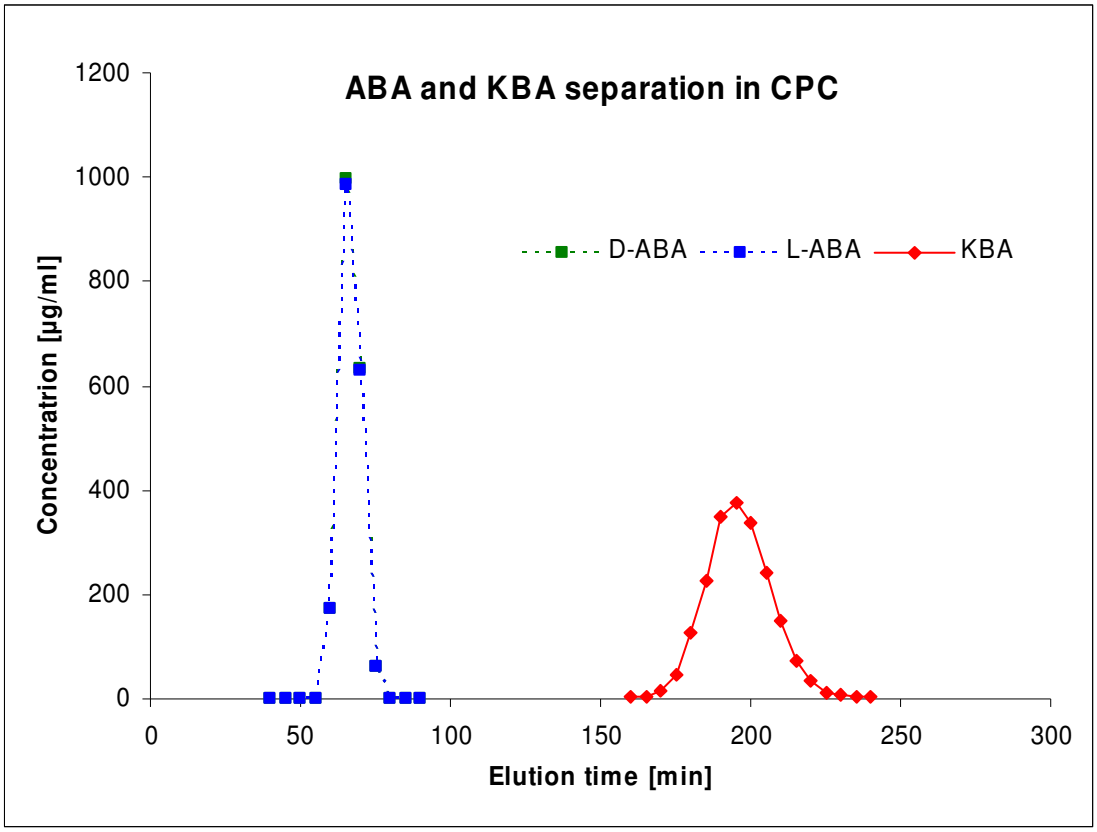


Figure 70 Resolution of ABA and KBA in CPC. The phase system was 14/14% PEG1000/potassium phosphate. DL-ABA (87mg) and KBA (43.5mg) were injected on the equilibrated column. The stationary phase displaced was 135ml. The mobile phase was the lower phase pumped at 5ml/min in descending mode. The coil was rotating at 2000rpm. CPC was operated at room temperature (20°).

There is a large separation between the peaks. The resolution factor is 4.29, 4 times greater then the Milli CCC (5.2.1).

5.2.3.2. Bioconversion and separation using CPC

Having achieved a better resolution of ABA and KBA in CPC (5.2.3.1) than in CCC (5.2.1), two experiments were done when both CPC and CCC were used as bioreactors in order to compare the two instruments. The D-ABA conversion ratio, as well as the resolution between L-ABA and KBA, were compared for the two machines. It has to be noted that the CPC (429ml) coil volume is 9.5 times bigger than the CCC "Milli"; (45ml) coil. To get comparable results, the substrate injection amount and the flow rate were scaled up 9.5 and 10 times, respectively (Table 40). The substrate injection amount per coil volume was the same for both CCC and CPC (0.4mg DL-ABA per 1ml of a coil). Since all parameters, such as the enzyme

concentration, were either maintained or scaled up according to the coil size (substrate injection amount, flow rate) a difference in obtained D-ABA conversion is believed to be caused primarily by the different method of mixing in the two coils, not by the difference in their size, although it has to be noted that the scale-up of the CCC-bioreactor has not yet been proven.

	CCC	**CPC**	**Scale factor**
Coil volume [ml]	45	429	9.5
Sample loop volume [ml]	1.75	23.8	13.6
D/L-ABA amount [mg]	17.4	166.3	9.5
D/L-ABA / coil volume [mg/ml]	0.4	0.4	0
Enzyme in the stationary phase [v/v]	2/100	10/50	0
Flow rate [ml/min]	0.5	5.0	10
Rotational speed	1800	2000	n/a
Temperature	20℃	20℃	n/a
D-ABA conversion [%]	56	12	

Table 40 Parameters of the coil size, injection amount and flow rate used for the comparison between the CCC and CPC. In the last row there are conversion ratios of D-ABA obtained in the CCC-bioreactor and CPC-bioreactor.

It also needs to be noted that, compared to the previous example of a successful bioconversion (5.2), this time a larger substrate amount was used. Based on Figure 53 (section 4.3.1), by increasing the substrate injection amount in CCC from 8mg to 17.4mg the total D-ABA conversion ratio should have reduced to about 50%. If the previously injected substrate amount guaranteeing 100% conversion was applied, it would not be easy to determine how much better is the conversion ratio in the CPC, as the conversion ratio in both cases would be 100%. So the conditions were deliberately chosen to give approximately 50% conversion ratio.

Analysis of the result showed that the D-ABA conversion ratio achieved in the CCC was 56%. The D-ABA conversion in CPC was only 12%, much poorer than the

conversion in CCC. In Figure 71, chromatograms obtained for the two experiments are overlaid.

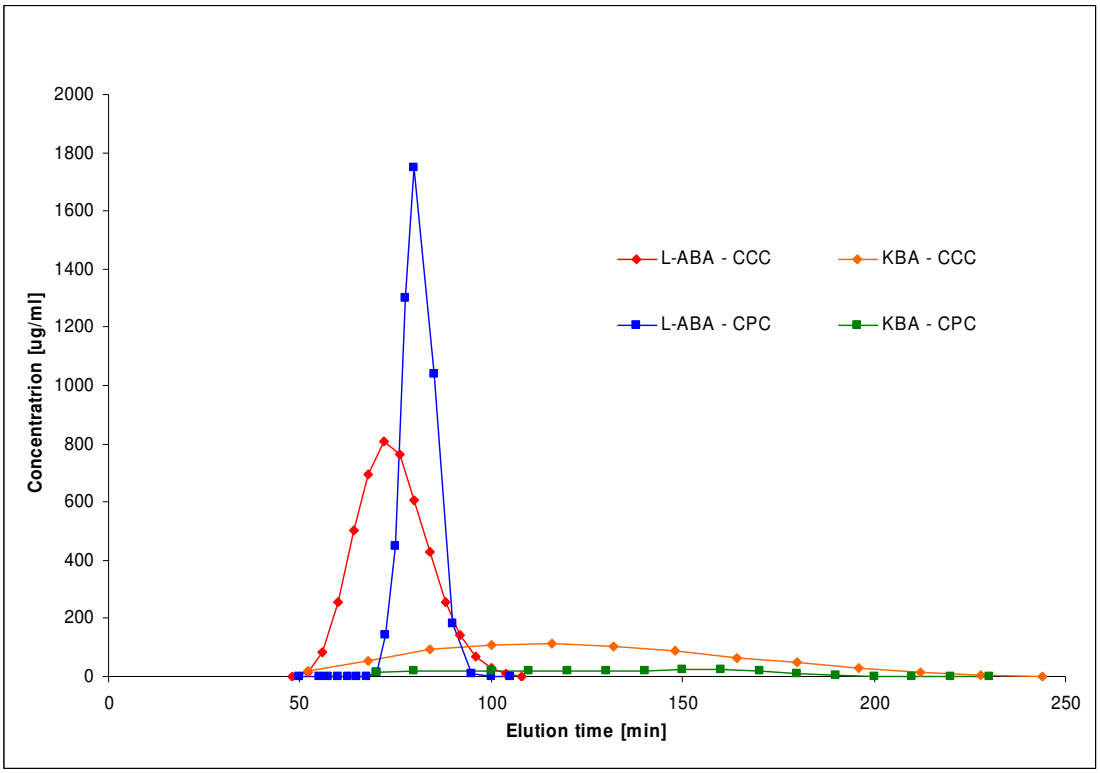


Figure 71 Comparison between ABA and produced KBA in the CCC and CPC in bioreactor-separator mode.

The difference between L-ABA peak shape eluting from CCC and CPC is noticeable. The peak for L-ABA which elutes from the CPC is more than twice higher and much narrower than the peak obtained for CCC. That is presumably caused by the more effective mixing characteristic for CPC. However as mentioned before (section 4.3.5; Figure 60), this might not be desired for the bioconversion. In CPC, D-ABA travels through the column as a narrow and very concentrated band contacting only a small amount of the enzyme. Even if the enzyme was distributed equally in the whole coil, only a small portion, that which was in contact with the substrate, was working. In the case of CCC, the substrate was migrating through a coil as a broader, less concentrated band involving more enzyme. Thus relatively more ABA could be converted in CCC.

Due to the low D-ABA conversion achieved in CPC, the technique does not seem to be a suitable tool for the reactor-separator.

5.3. Bioconversion and separation using Multiple Dual-Mode (MDM)

Although applying the standard CCC mode to the bioreactor allowed D-ABA to be totally converted, the resolution between L-ABA and KBA was not satisfactory (Figure 69). Furthermore, the conversion of D-ABA in CPC was not complete (Figure 71). Resolution (R_S) in CCC increases with the length of the coil (L); $R\alpha\sqrt{L}$. Thus one approach to improve the resolution in the CCC would be to increase the length of the CCC coil. To obtain a complete resolution (R_S=1.25) under the condition shown in Figure 69, it was calculated that the coil length would need to be increased from 8 to 100m. This would be impossible to wind on the small bobbin of the Milli CCC. An alternative way to increase the effective length of a coil was applying a Multi Dual – Mode of operation.

Both the mobile and the stationary phases in CCC are liquid, therefore it is possible to reverse the elution mode, such that the initial mobile phase becomes the stationary phase and vice versa as shown in Figure 72. This allows the column length to be effectively increased, improving the resolution on the same instrument. This is called Multiple Dual-Mode and can be performed on a standard Milli CCC.

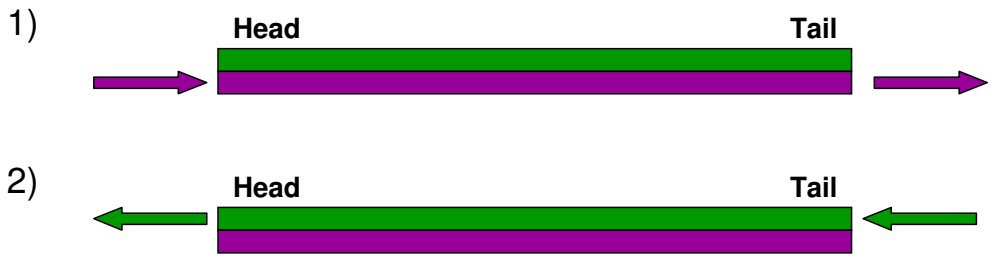


Figure 72 Multi Dual-Mode. Both the mobile phase as well as the flow direction are altered when one of separated compounds is about to elute.

5.3.1. Retention studies done on Milli CCC using the ATPS 14/14% PEG1000/potassium phosphate

Prior to using the Milli CCC in Multiple Dual-Mode, retention studies were performed using the ATPS 14/14% PEG1000/potassium phosphate. That was done to obtain a better understanding of the behaviour of this system in CCC. Three parameters were varied: the stationary phase (upper and lower phase), direction of coil

rotation (clockwise or counter clockwise) and flow direction (head to tail or tail to head), which in total makes eight combinations.

Figure 73 shows the volume of stationary phase displaced plotted against the square root of the flow rate for each of the tested possibilities. The less stationary phase displaced, the more the stationary phase in the coil which means better resolution between peaks.

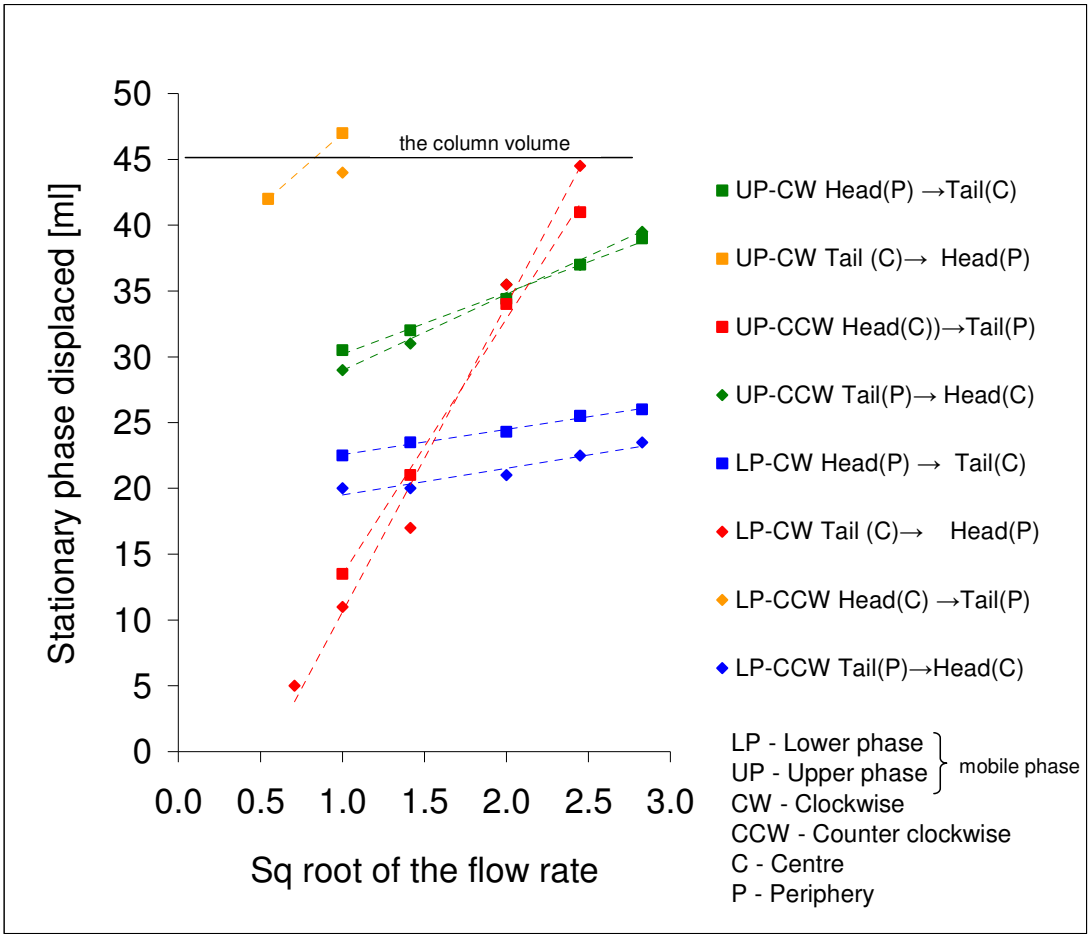


Figure 73 Retention studies of ATPS made of 14/14% PEG1000 and potassium phosphate tested in "Milli" CCC, 45.1ml coil volume, 2.7mm bore. The coil was first filled with a phase. The coil was rotated at 1800rpm and the mobile phase was pumped through at a low flow rate till equilibrium was achieved, which meant that no more stationary phase eluted from the coil. The volume of the stationary phase displaced was recorded. The flow rate was increased and the volume of further stationary phase displaced was recorded.

The PEG-rich upper phase, where the enzyme partitions, has to be used as the initial stationary phase which makes the lower, salt-rich phase the mobile phase. Based on the results presented in Figure 73, either pumping the lower phase from head to tail with the coil rotating in a clockwise direction, or from tail to head in the

counter clockwise direction, guarantees good stationary phase retention. Although there was little SP displaced when the MP was pumped from tail to head with the coil rotating in clockwise direction, the system was sensitive to changes in mobile phase flow rate. A slight increase in flow rate resulted in a greater volume of SP displaced compared to the two previously described conditions.

Out of the two highlighted conditions from Figure 73, pumping the lower phase from head to tail with the coil rotating in a clockwise direction was not applied. This is because after switching the flow mode in MDM, the stationary phase would not have retained in the coil when the upper mobile phase is pumped from tail to head. Therefore, the Multiple Dual-Mode operating conditions selected were: the coil rotated in a counter clockwise direction, the initial mobile phase as the lower phase pumped from tail to head, to be altered to the upper phase pumped from head to tail direction.

5.3.2. Separation of ABA and KBA in Multiple Dual-Mode

Before performing the CCC-bioreactor in Multiple Dual-Mode, first the resolution of ABA and KBA under these conditions was tested. The mixture of ABA and KBA was injected with the mobile, lower phase. Then, when ABA was about to elute, both the mobile phase and the flow direction were altered so the upper phase became the mobile phase. That mode was held for 40min at which time the conditions were returned the initial conditions, so again the lower phase was the mobile phase. Fractions were collected every 4min and analysed on HPLC for ABA and KBA.

The solvent system used was an aqueous two-phase system: 14/14% PEG1000/ potassium phosphate, the stationary phase was the upper, PEG-rich phase. The mixture of DL-ABA (8.15mg) and KBA (4.23mg) was injected with the mobile (lower, salt rich) phase, pumped at 0.5ml/min in tail to head direction. After 45min, when ABA was about to elute, both the mobile phase and the flow direction were altered and for the next 40min the upper PEG rich phase was pumped as the mobile phase from head to tail. After that time the operating mode was brought to the initial conditions so that the mobile phase was the lower, salt rich phase. All that time the coil was rotating in a counter clockwise direction at 1800rpm. The temperature of the coil was 20°C. A reconstructed chromatogram is presented in Figure 74.

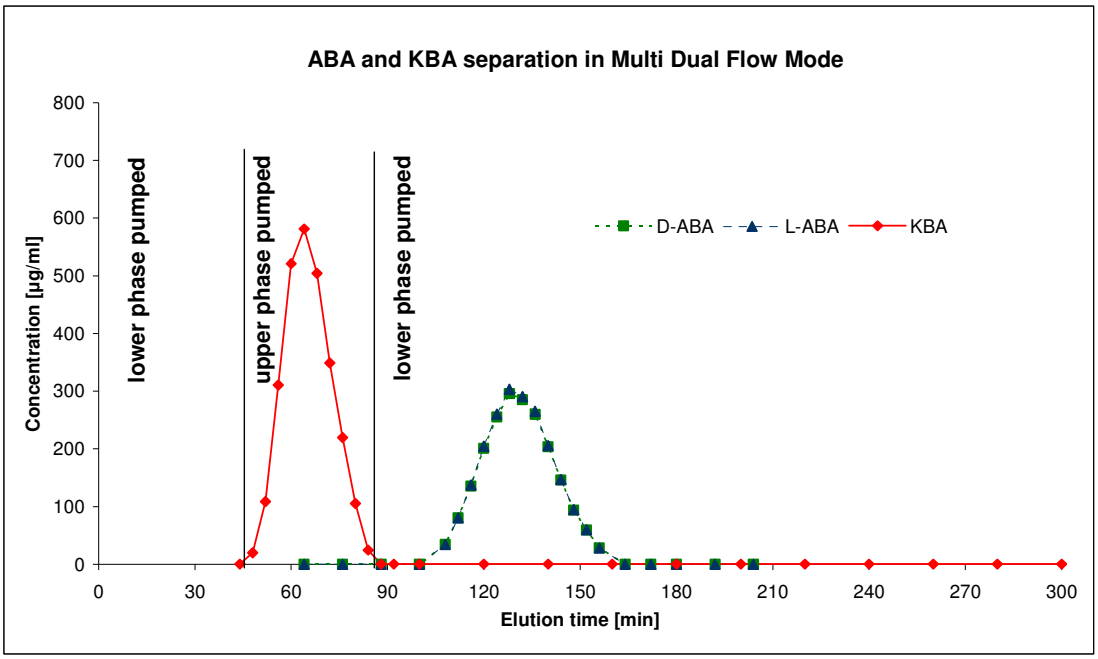


Figure 74 Separation of ABA and KBA in Multiple Dual-Mode. The chromatogram was reconstructed based on HPLC analysed fractions eluted from CCC.

ABA and KBA are totally separated with the resolution factor of 1.59. This is higher than 1.03, obtained for the standard Single Flow-Mode used on the same machine (5.2.1). Thus, applying MDM to the 8m coil allowed separation of the two peaks with a resolution which, in Single Flow Mode, would have required a 19m long coil.

5.3.3. Performing the CCC bioconversion in Multiple-Mode

Having achieved a successful separation in Multiple Dual-Mode, the next step was performing a CCC-bioreactor in this mode. The coil was initially filled with the stationary phase, which was the upper, PEG-rich phase with the enzyme (2ml enzyme supernatant in 100ml of the stationary phase). Then the mobile phase which initially was the lower, salt-rich phase was pumped in the tail to head direction through the coil rotating at 1800rpm in a counter clockwise direction. The flow rate was 0.5ml/min. The substrate DL-ABA was injected in the amount of 7.98mg with the mobile phase. When ABA was about to elute (45min), both the mobile phase and the flow direction were altered. Thus the upper phase was pumped the from head to tail direction for the next 40min, after which the flow mode was brought back to the

initial conditions. Fractions taken every 3min were analysed on HPLC for ABA and KBA.

An aqueous two-phase system, 14/14% PEG1000/potassium phosphate, was used as the solvent system. The temperature was 20°C. A reconstructed chromatogram based on the HPLC analysis is presented in Figure 75.

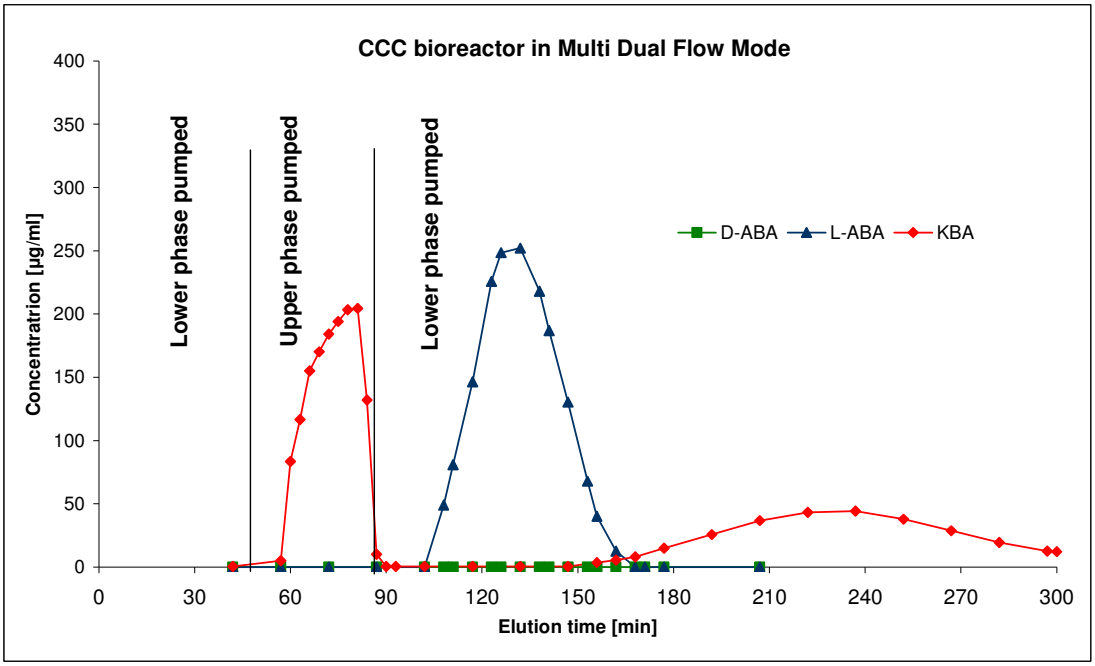


Figure 75 Bioconversion and separation in CCC – Multiple Dual-Mode Chromatogram reconstructed based on HPLC analysed fractions eluted from CCC.

There was a total conversion of D-ABA: there was no D-ABA present in any of the fractions. L-ABA is also totally separated from the KBA (Figure 75), which was produced inside the column. Thus total conversion and total separation has been achieved and a successful, working CCC-bioreactor/separator has been created.

5.4. Comparison between three different bioreactor modes

In Table 41 the three ways of isolating L-ABA using CCC are compared. In the first column "Conversion and separation" the bioreaction was performed in a test tube and only the separation of the reaction mixture performed by CCC. The experimental conditions were as follows: to 46.6mg DL-ABA dissolved in 10ml ATPS (14/14% PEG1000/potassium phosphate) was added 200µL of the enzyme. The reaction was

carried out for 5h till the deracemisation was completed (confirmed on HPLC). The ability of CCC to separate ABA and KBA had been already demonstrated (Figure 67).

The last two columns of Table 41 refer to the CCC-bioreactor performed in Single Flow Mode (5.2) and Multiple Dual-Mode (5.3.3).

As can be seen in Table 41, key parameters such as the enzyme concentration in the stationary phase, substrate injection amount, flow rate, the phase system and temperature were the same. What differs for these experiments is the separation time, the enzyme consumption and the achieved resolution between ABA and KBA. The poorest resolution (0.35) was obtained when the CCC-bioreactor was performed in Single Flow Mode. Complete separation was achieved when the CCC-bioreactor was performed in the Multiple Dual-Mode and when the test tube reaction mixture was simply separated on the CCC. In the first case, the enzyme consumption was relatively high: 1.4ml of the enzyme supernatant was used. This technique requires more enzyme than the Single Flow Mode (1ml) as during the reverse phase mode the upper phase with the enzyme was pumped as the mobile phase. Much less enzyme was needed to convert the same amount of D-ABA in a test tube. Only 35µl of the enzyme would have been used to convert 4mg of D-ABA in a volume of 1.72ml, which was the volume of the CCC sample loop. Armstrong *et al*[140] have reported that much lower enzyme consumption was achieved by performing the reaction in an external chamber, rather than in CPC where the enzyme was distributed in the whole column volume.

However, performing the reaction and separation in a single step, with Multiple Dual-Mode, took 300min, whereas the separate conversion and purification required 300min to complete the deracemisation and another 250min for the CCC separation, which in total makes 550min, nearly twice as long.

		Conversion and separation	CCC-bioreactor Single Flow Mode	CCC-bioreactor Dual Flow Mode
Time [min]	**Bioconversion**	300	250	300
	Separation	250		
	Total time	**550**	**250**	**300**
Enzyme used for the reaction [µl] / stationary phase volume [ml]		**200**/10ml	**1000**/50ml	**1400**/70ml
enzyme used per mg of product produced [µl]		**50**	**250**	**350**
Enzyme activity by the colorymetric assay (dilution 1:100) [abs/min]		0.275	0.295	0.294
D/L ABA in 10ml of ATPS [g]		0.0466	0.0465	0.0464
Injection volume [ml]		1.72	1.72	1.72
D/L ABA injecion amount [mg]		8	8	8
Rotational speed [rpm]		1800	1800	1800
Rotational direction		Counter clockwise	Clockwise	Counter clockwise
Flow rate [ml/min]		0.5	0.5	0.5
CCC temperature [°C]		22	22	22
D-ABA conversion ratio [%]		100	100	100
Resolution between ABA and KBA		1.05	0.35	0.97
Solvent system		ATPS 14/14 PEG1000/ potassium phosphate		

Table 41 Parameters for the three experiments where pure L-ABA was isolated: CCC-bioreactor performed in a Single Flow Mode, CCC-bioreactor performed at the Multiple Dual-Mode, the reaction mixture after completed deracemisation separated on the CCC.

5.5. Conclusion

This chapter presents the first successfully created, optimised CCC-bioreactor using a model process of isolating enantiopure L-ABA from the racemic mixture of ABA.

Although in the literature[140,138,139,141,142] there are examples of applying countercurrent chromatography as a bioreactor/separator, none were discovered which would perform a total bioconversion of an injected substrate by the enzyme immobilised in the stationary phase, as well as a complete separation of the products created in the column.

By selecting the conditions optimized in Chapter 5, L-ABA of 99% enantiomeric activity was achieved totally separated from KBA. To obtain a total separation of unaltered L-ABA from the KBA produced in the coil, Multiple Dual-Flow mode was applied.

In spite of more effective mixing, the hydrostatic CCC (CPC) was found to be a less effective tool as a CCC-bioreactor (6.2.2.2.).

6. ENZYME IN WHOLE CELLS

6.1. Introduction

In spite of the successfully performed CCC-bioreactor, with L-ABA totally isolated from D-ABA (5.3.3.), the enzyme instability made using it in a continuous mode impossible. In fact, even performing a second injection of the substrate would result in a decline in purity (4.3.10.).

It was considered that by using the enzyme in whole cells of *E. coli*, the enzyme's stability during the bioconversion might be improved considerably. Therefore, it was investigated if the enzyme in whole cells can be applied to the CCC-bioreactor.

E. coli cells were used to express the enzyme, DAAO. In the previous sections of this thesis, the free enzyme (2.4.2.1.) was isolated from cells by mechanical disruption and used for the CCC-bioreactor. Since the *E. coli* membrane is permeable to ABA and KBA, it is possible to perform a bioconversion using whole cells instead of the free, isolated enzyme. In this case, the substrate (D-ABA) migrates through the membrane to the cytoplasm to be converted there to KBA. Since the enzyme is encapsulated in whole cells, it is separated from the reaction medium. This reduces the inactivation of the enzyme and might improve its stability in the phase system used for the CCC-bioreactor.

Moreover, that opens up the possibility of application of a wide rage of aqueous-organic two-phase systems, which are the most widely used phase systems for CCC but known to inactivate a free enzyme (section 3.4). Therefore, in addition to DAAO, *E. coli* cells with another enzyme activity, mono amino acid oxidase, MAO (section 1.5.4), suitable for catalysing the oxidation of nonpolar substrates such as α-methylbenzylamine, amylamine and 2-phenylpyrrolidine, were tested. Being nonpolar those substrate molecules would distribute between the two phases of an organic two-phase system.

In the literature there are examples of using enzymes in whole cells of microorganisms for industrial production of such chemicals as L-sorbose, L-malic acid, D-aspartic acid and 2-keto-L-gulonic acid.[168]

Sanchez and Demain [169] in their review published in 2011 reported the use of whole cells of various microorganisms with cloned genes encoding feruloyl-CoA synthetase and enoyl CoA hydratase for the production of vanillin from ferulic acid.

Microorganisms used were, *E. coli*, *Bacillus pumilis, Bacillus fusiformis* and *Pseudomonans putida.* In turn, Hua *et al*[170] used *Pseudomonans sp* to convert ferulic acid to vanillin. Dhillon *et al*[171] (1987) used *E. coli* with the activity of glucosamine synthase to convert fructose-6-phosphate into glucosamine-6-phosphate.

Abad *et al*[172], using D-methionine as the substrate, demonstrated a three-fold enhanced stability of *Trigonopsis variabilis* D-amino acid oxidase (TvDAAO) in whole cell of *Pichia pastoris* in comparison to the free form of this enzyme expressed in *E. coli*. 49% of the total intracellular oxidase activity was available after applying permeabilisation of *P. partoris* with 10% propanol.

6.2. Whole cells of *E. coli*

Two types of whole cells of *E. coli* were obtained from Ingenza. These differed in activity. One type had the enzymatic activity of DAAO (1.5.3), whereas the other had MAO activity (1.5.4).

Cells of *E. coli* were obtained in frozen form, defrosted to dispense separate quantities into Eppendorf vials and then stored frozen at -80°C. Before analysis, the cells were thawed by placing an Eppendorf vial into cold water.

6.3. Whole cells of ***E. coli*** with MAO activity

Monoamine oxidase (MAO) catalyses the oxidative deamination of primary amines (section 1.5.4). The specific reaction examined was the oxidation of α-methylbenzylamine to acetophenone. Since substrate and product, are nonpolar, a range of potential organic two-phase systems was tested for the CCC-bioreactor with this enzyme.

The enzymatic activity of MAO was also tested against two substrates; 2-phenylpyrrolidine and amylamine.

6.3.1. Activity against α-methylbenzylamine

The reaction was performed in the presence of 50mM sodium phosphate buffer, pH 7.6, which was optimal for the enzyme. 120mg of *E. coli* cells with MAO activity and 10µl of α-methylbenzylamine was placed into 2ml of the sodium phosphate buffer. The reaction was monitored by HPLC analysis of a sample with the gradient presented in Table 42.

Figure 76 shows a chromatogram of a sample analysed 21.5h after the reaction was initiated. In contrast to the impression obtained from analysing the chromatogram, the amount of produced acetophenone is very little. For comparison, Figure 77 shows a chromatogram obtained for equal amounts of α-methylbenzylamine and acetophenone injected onto HPLC. Due to the presence of a carbonyl group, at the same molar concentration, acetophenone gives a much stronger signal than α-methylbenzylamine. This comparison shows that the reaction yield in Figure 76 was very poor.

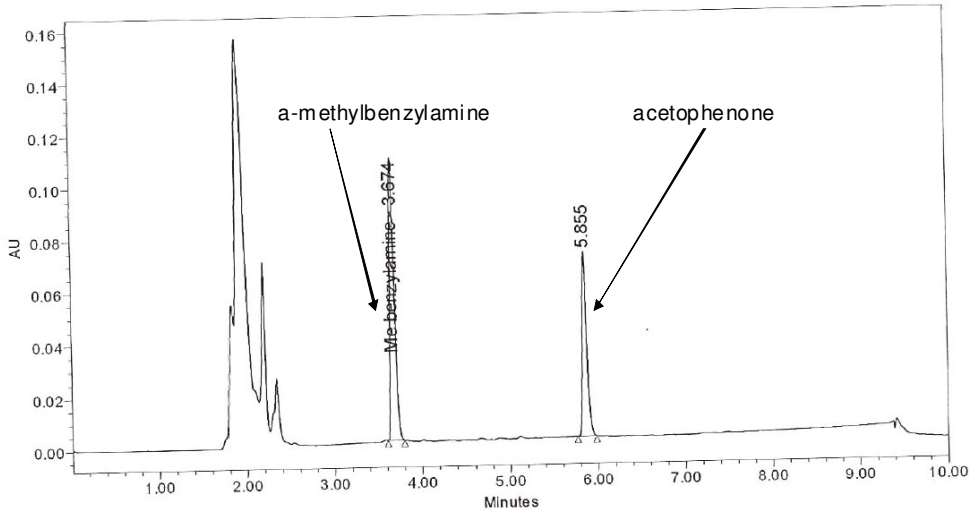


Figure 76 Conversion of α-methylbenzylamine and acetophenone after 21.5h, catalysed by 120mg of cells, analysed by HPLC on a C18 column (GraceSmart) using the method presented in Table 41. Detection at 257nm.

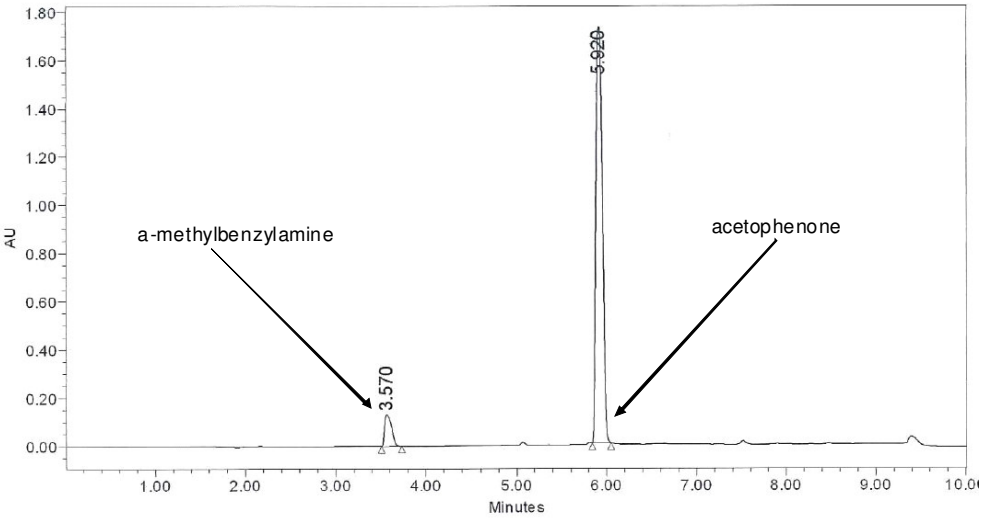


Figure 77 Analysis of α-methylbenzylamine and acetophenone on HPLC, C18 column (GraceSmart). Concentration of both chemicals was 1µl/ml of water. Detection at 257nm (maximum for α-methylbenzylamine)

	Time [min]	Flow [ml/min]	%A	%B	%C	%D	Gradient
1		1.00	95.0	5.0	0.0	0.0	
2	7.00	1.00	5.0	95.0	0.0	0.0	Linear
3	7.10	1.00	95.0	5.0	0.0	0.0	Linear

Table 42. The HPLC method used for α-methylbenzylamine and acetophenone analysis. Line A: water, Line B: acetonitrile

6.3.2. Activity of MAO in the whole cells in organic solvents

Despite poor conversion of α-methylbenzylamine an in aqueous environment (6.3.1), the MAO activity of the whole cells of *E. coli* was tested in 12 systems (Table 43) made of water and organic solvents such as heptane, ethyl acetate, methanol and butanol. Since all 12 phase systems exist as two-phase systems, they could be used as a phase system for CCC.

Into 2ml of a phase system, 36.9mg of cells and 20µl of α-methylbenzylamine was placed.

The results form the experiment showed that there was no acetophenone produced, even 20 hours after the incubation was started.

System No.	Solvent [µl]				
	Heptane	EtOAc	MeOH	Butanol	Water
1	0	0	0	1000	1000
2	0	400	0	600	1000
3	0	800	0	200	1000
4	0	1000	0	0	1000
5	145	855	145	0	855
6	250	750	250	0	750
7	400	600	400	0	600
8	545	455	545	0	455
9	715	285	715	0	285
10	835	165	835	0	165
11	950	50	950	0	50
12	1000	0	1000	0	0

Table 43. Composition of phase systems made by the liquid-handling robot (6.4.3.3), to test MAO activity in the whole cells of *E. coli*.

6.3.3. Activity of MAO in the whole cells against various substrates.

Since the MAO activity in the whole cells against α-methylbenzylamine was very low, two other substrates were tested, amylamine and 2-phenylpyrrolidine. For the comparison α-methylbenzylamine was also used.

The activity of MAO was determined by the modified colorimetric assay presented in section 2.5.2.1. Since the enzyme activity was compared against three different substrates, each set of three wells, as the analysis was preformed in triplicate, contained one of the three different substrates at the same molar concentration. Then, using a 12 channel automatic pipette, a mixture of enzyme assay solution with *E. coli* cells was added. The absorbance was measured at 490nm in 2min intervals.

Results are presented in Figure 78. Among the three tested substrates, the activity of MAO was the lowest towards the initially tested (6.3.1) α-methylbenzylamine. In comparison to methylbenzylamine, 12 times more 2-phenylpyrrolidine was converted when DAO was used in the whole cells. The difference in activity might have resulted from substrate specificity of the enzyme as well as the selective permeability of the cell membrane towards the tested substrates. To eliminate the last possibility, the activity of the free, isolated enzyme towards the three substrates would need to be tested, which was done and presented in the next section.

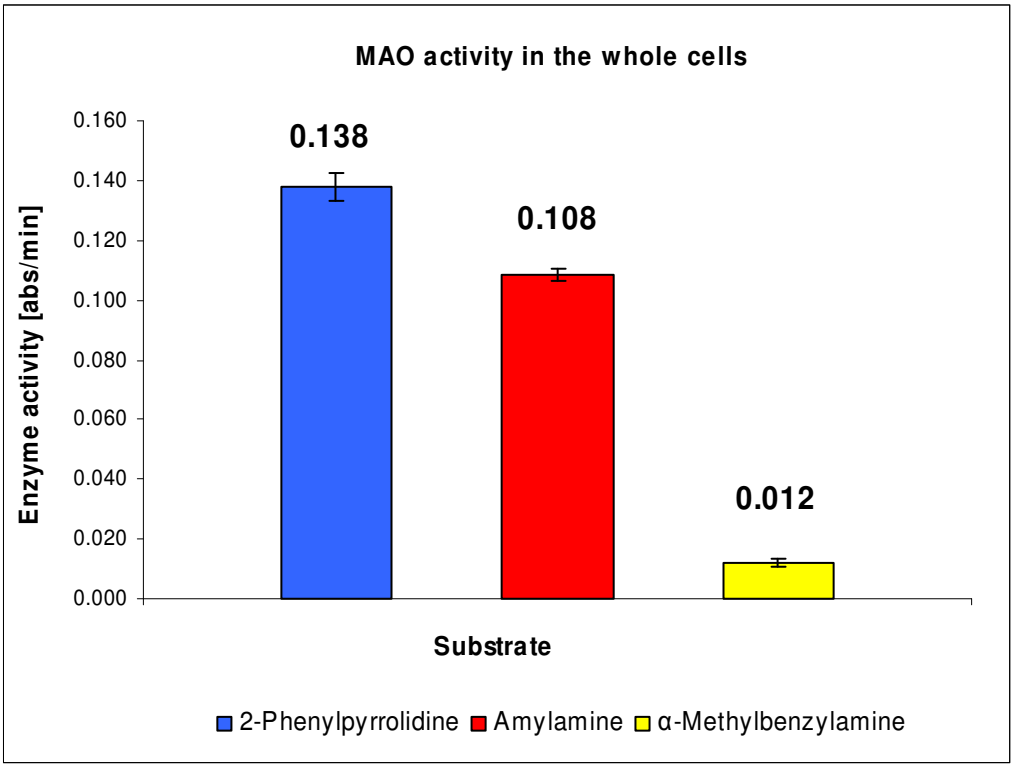


Figure 78. Activity of MAO in the whole cells of *E. coli* against three substrates: 2-phenylpyrrolidine, amylamine and α-methylbenzylamine. To each well of a 96-well plate 20µl of a substrate solution (0.1M) was added. Then 180µl of the enzyme solution containing *E. coli* cells (930µg). An equivalent of 1ml OD_{600}=5 cells was suspended in 322µl of water. 200µl of that suspension was mixed with 3.4ml of the enzyme assay solution.

6.3.4. Activity of the free form of MAO against various substrates

The activity of MAO in the whole cells (6.3.3) seemed to be too low to use in the CCC-bioreactor. Therefore, the activity of the free enzyme, isolated from *E. coli* was tested against the same three substrates. The experiment was done as the *E. coli* cell membrane was suspected to affect permeability of substrates and therefore affect the activity of MAO.

In order to do a direct comparison between the activity of MAO in the whole cells and its free form, the same amount of cells, as used in the previous experiment (6.3.3) was lysed (section 2.4.2.2) and the supernatant containing the free enzyme protein used for the assay. Thus into each well of a 96-well plate was placed 20µl of a substrate solution (0.1M) and 180µl of the enzyme solution containing MAO isolated from 930µg of *E. coli.*

As presented in Figure 79, the free form of MAO is the most active against 2-phenylpyrrolidine and amylamine and least towards α-methylbenzylamine, just as it was in the cells (Figure 78). The enzyme activity in the whole cells against these substrates were lower by only 15, 8 and 29%, respectively. Therefore, using the enzyme in the whole cells and in the isolated form did not seem to make a significant difference in the activity towards analysed substrates. Thus, the low activity of the enzyme did not result from the presence of the cell membrane.

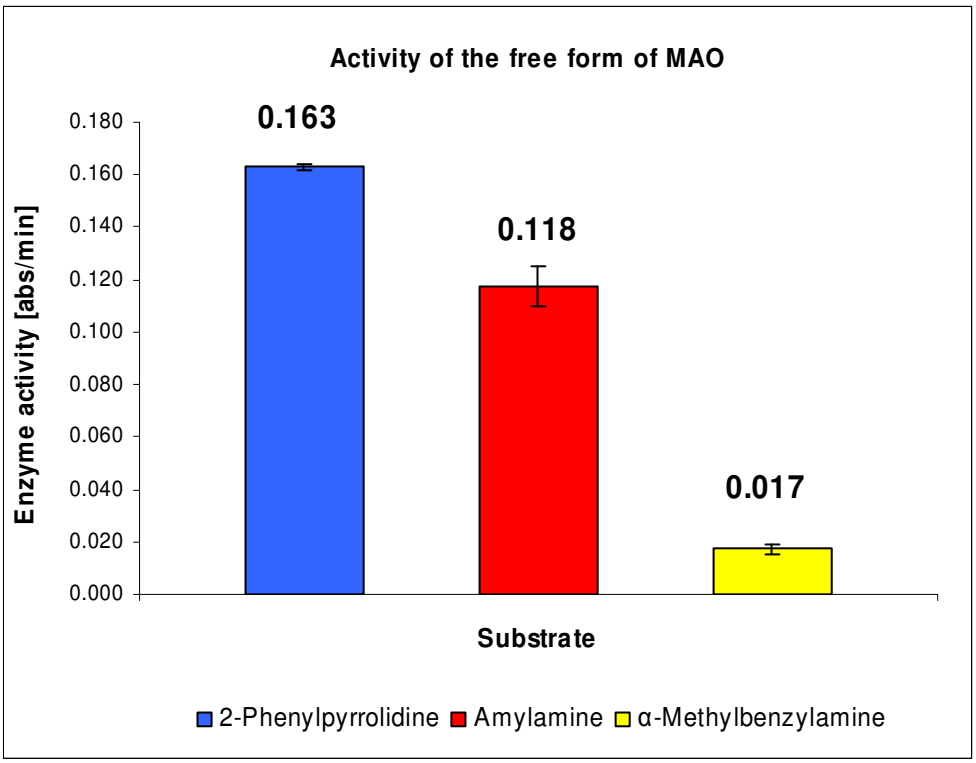


Figure 79. Activity of the free form of MAO in isolated from *E. coli* cells, against three substrates; 2-phenylpyrrolidine, amylamine and α-methylbenzylamine.

6.3.5. Conclusion to MAO

Due to a very low activity of MAO in the obtained whole cells of *E. coli*, no CCC experiment was performed. In comparison to the whole cells of *E. coli* with DAAO activity (section 6.4), the activity of MAO was 180 times lower. It meant that to convert the same amount of molecules as *E. coli* cells with DAAO activity, 180 times more cells with MAO activity needed to be used. *E. coli* cells were found to flocculate and block CCC (6.4.3.4). Therefore, using 180 times more concentrated cell suspension in CCC-bioreactor seemed to be a big limitation.

6.4. Whole cells of *E. coli* with DAAO activity

6.4.1. Determining the D-ABA conversion ratio

The chiral ABA derivatisation used in the HPLC analysis of ABA was previously discovered to stop the enzymatic conversion of D-ABA catalysed by the free form of D-amino acid oxidase (2.5.1.2d). For accurate analysis it had to be shown that the derivatisation step also stops the bioconversion catalysed by the enzyme in whole cells. Furthermore, the derivatised sample needs to be centrifuged before injecting on an HPLC column to avoid column blockage caused by cells. Therefore, it was also investigated how quickly the sample has to be centrifuged after a derivatisation, as it might happen that only removing cells from a reaction mixture stops the reaction.

The reaction mixture was made by placing 10.6mg of whole cells into 2ml of 50mM potassium phosphate buffer, pH 7.5, and adding 100µL solution of DL-ABA (3.1mg). 500µL of the reaction mixture was immediately derivatised with the derivatising reagent premixed with borate buffer, keeping all proportions as desctibed in section 2.5.1.2b. To investigate how time affects a derivatised sample before centrifugation, the derivatised sample was divided into 5 fractions and each of them was centrifuged under the same conditions after a different elapsed time. The first fraction was centrifuged immediately (5min, 10000 rpm) after the derivatisation and analysed on HPLC. Next fractions were centrifuged in sequence of 8 min intervals and analysed on HPLC. For the control, the rest of the reaction mixture was derivatised after 32min, immediately centrifuged and analysed on HPLC. Results are presented in Table 44.

	Time after which the derivatised sample was centrifuged [min]	Peak area [mVs]		Ratio D/L
		L-ABA	D-ABA	
Derivatised reaction mixture	0	1679	1640	0.98
	8	1692	1654	0.98
	16	1691	1665	0.98
	22	1686	1659	0.98
	28	1689	1664	0.99
Control	0	1938	230	0.12

Table 44 Demonstration that the chiral derivatisation method stops the enzymatic action of DAAO in whole cells in D-ABA. HPLC peak area of D-ABA and L-ABA obtained for the derivatised enzymatic solution.

The ratio D- to L-ABA was constant (0.98) in all analysed samples. The time after which the derivatised sample was centrifuged did not affect the D-ABA conversion ratio. This means that the bioconversion was stopped by adding the derivatisation reagent to the sample. The conversion ratio of 0.12 obtained for the control incubated for 32min before the derivatisation proves that the enzyme in whole cells was active.

This experiment shows that the ABA derivatisation method, described in section 2.5.1.2b, totally stops the enzymatic action of the enzyme in whole cells.

6.4.2. Whole cells in organic solvents

The activity of DAAO in whole cells was tested in 36 organic solvents systems developed by Dr Ian Garrard for the collaborative project on "Anti-angiogenic activities of the volatile oil of *Angelica sinensis*"[160] used already in section 3.3. All the 36 solvent system were made by liquid-handling robot (Perkin Elmer), using 12 organic solvents and water.

6.4.2.1. Activity in organic solvents

To each 5 ml test tube containing 2ml of each phase system made by the robot, 10µl DL-ABA (1.4mg/ml) solution was added. Next the enzymatic reaction was initiated by adding 20µl of suspension containing 10.0mg whole cells to every test tube at 30 sec interval. A rotating wheel provided continuous phase mixing during the reaction. The bioconversion was stopped after 30 min (timer) by derivatisation of the sample (2.5.1.2). Derivatised samples were analysed on HPLC.

The enzyme activity was expressed as a percent of converted D-ABA after 30 min reaction (Table 45), using L-ABA as an internal standard. A higher value, showing greater D-ABA conversion, indicated higher enzymatic activity in a system. In the last column, enzyme activity is shown using different colours. A green colour shows very high enzyme activity, whereas red indicates very low. The highest conversion (over 60% converted D-ABA) was obtained for systems No 3, 4, 5, 6 and 27. For those system and systems No 17 and 31, where the conversion was above 30%, the enzyme stability was determined (6.4.2.2).

System No.	Solvent [µl]													Peak Area [mVs]		D-ABA conversion [%]
	Heptane	EtOAc	MeOH	Butanol	Water	ACN	Toluene	Acetic Acid	Acetone	tBME	DCM	Ethanol	Propanol	L-ABA	D-ABA	
1	0	0	0	1000	1000	0	0	0	0	0	0	0	0	436	357	18
2	0	400	0	600	1000	0	0	0	0	0	0	0	0	559	417	25
3	0	800	0	200	1000	0	0	0	0	0	0	0	0	609	246	60
4	0	1000	0	0	1000	0	0	0	0	0	0	0	0	551	145	74
5	145	855	145	0	855	0	0	0	0	0	0	0	0	530	83	84
6	250	750	250	0	750	0	0	0	0	0	0	0	0	599	232	61
7	400	600	400	0	600	0	0	0	0	0	0	0	0	588	478	19
8	545	455	545	0	455	0	0	0	0	0	0	0	0	548	466	15
9	715	285	715	0	285	0	0	0	0	0	0	0	0	549	529	4
10	835	165	835	0	165	0	0	0	0	0	0	0	0	521	521	0
11	950	50	950	0	50	0	0	0	0	0	0	0	0	589	587	0
12	1000	0	1000	0	0	0	0	0	0	0	0	0	0	399	397	0
13	1000	0	900	0	0	100	0	0	0	0	0	0	0	475	473	0
14	1000	0	600	0	0	400	0	0	0	0	0	0	0	552	548	1
15	1000	0	0	0	0	1000	0	0	0	0	0	0	0	0	0	
16	1000	0	0	0	0	900	100	0	0	0	0	0	0	143	128	10
17	1000	0	0	0	0	600	400	0	0	0	0	0	0	14	8	44
18	0	0	0	950	950	0	0	100	0	0	0	0	0	248	240	3
19	0	0	0	900	900	0	0	200	0	0	0	0	0	60	49	18
20	800	0	560	0	240	0	0	0	0	0	400	0	0	559	562	-1
21	0	0	0	400	1000	200	0	0	0	400	0	0	0	549	524	5
22	625	250	500	0	0	625	0	0	0	0	0	0	0	198	187	6
23	0	0	0	842	1052.5	0	0	0	105.5	0	0	0	0	530	510	4
24	333	333.5	0	0	333.5	0	0	0	1000	0	0	0	0	390	346	11
25	1000	0	800	0	0	0	0	0	200	0	0	0	0	392	389	1
26	0	0	0	400	1000	200	0	0	0	400	0	0	0	497	471	5
27	0	0	0	0	1000	0	0	0	0	1000	0	0	0	555	162	71
28	0	0	800	0	533.5	0	0	0	0	0	666.5	0	0	251	249	1
29	1000	0	0	0	0	800	0	0	0	0	200	0	0	20	16	20
30	750	0	0	0	625	0	0	0	0	0	0	625	0	595	467	22
31	513	513	0	0	307.5	0	0	0	0	0	0	666.5	0	580	370	36
32	0	800	0	0	800	0	0	0	0	0	0	400	0	552	409	26
33	0	500	0	800	600	0	0	0	0	0	0	100	0	619	459	26
34	0	0	0	889	889	0	0	0	0	0	0	222	0	574	518	10
35	1000	0	0	0	750	0	0	0	0	0	0	0	250	684	625	9
36	1143	0	457	0	114.5	0	0	0	0	0	0	0	285.5	518	495	4
Water					2000									607	62	90
Water					2000									617	56	91

D-ABA conversion [%]
>50
31-60
11-30
<10

Table 45. The enzyme activity 36 organic two-phase systems which are combinations of 12 different organic solvents and water. Peak areas of D- and L-ABA derivatised and analysed on the HPLC after 30 min bioconversion are shown. In the last column percent of unreacted D-ABA is given. The conversion ratio is marked with different colours. In green are marked systems with a very high conversion ratio - less than 50% D-ABA stayed unconverted. Whereas, in red colour are shown systems in which the conversion was very low, more than 90% D-ABA stayed unreacted.

6.4.2.2. Stability in organic solvents

For the 7 solvents systems mentioned above, where the remaining D-ABA was lower than 70%, the stability was determined by analysing the enzyme activity after 2 and 4 hours of whole cells incubation in the tested phase systems. The enzyme activity was expressed as % of converted D-ABA. The higher the value, the more D-ABA was converted and therefore the more active the enzyme must have been. Results are presented in Table 46.

For a better understanding of the effect of the tested phase systems on whole cell stability, the results from Table 46 are also presented in a graph (Figure 80).

The enzyme was most active and stable in system No 27, made of water/ *tert*-butyl methyl ether. 100% D-ABA was converted even if the whole cells had been incubated for 4 hours in that system. A high enzymatic activity was also noticed for system No 4 (ethyl acetate/water) where 78% D-ABA was converted. Even after 4 hours, 46% of the initial enzymatic activity remained.

It had previously been found that the free enzyme was also the most active in these two systems (see section 3.4). 100% D-ABA conversion was achieved when free enzyme was used in system No 27. However, it was found that these systems could not be used because of unfavourable ABA partitioning. Moreover, to provide a mixing between the two phases, a rotary wheel was used. By contrast, CCC provides considerably more efficient mixing between the phases. That would expose the enzyme in the aqueous phase to contact with the organic solvent to a higher extent and presumably inactivating it.

Among the tested aqueous-organic phase systems, there is not any which would provide both whole cell enzyme stability and preferable ABA partitioning. Therefore, a phase systems based on organic solvents was not selected for use within this project for the CCC-bioreactor with the whole cells of *E. coli*.

System No.	Solvent [µl]									Incubation time [h]	Peak Area [mVs]		D-ABA conv. ratio [%]	% of the initial activity
	Heptane	EtOAc	MeOH	Butanol	Water	ACN	Toluene	tBME	Ethanol		L-ABA	D-ABA		
Stand.										**-**	**6731**	**6894**	**-2**	**-**
Syst. No 3	0	800	0	200	1000	0	0	0	0	**0**	610	221	**64**	**100**
										2	639	591	**8**	**12**
										4	563	540	**4**	**6**
Syst. No 4	0	1000	0	0	1000	0	0	0	0	**0**	587	129	**78**	**100**
										2	550	263	**52**	**67**
										4	573	367	**36**	**46**
Syst. No 5	145	855	145	0	855	0	0	0	0	**0**	507	52	**90**	**100**
										2	645	479	**26**	**29**
										4	532	502	**6**	**6**
Syst. No 6	250	750	250	0	750	0	0	0	0	**0**	448	197	**56**	**100**
										2	1340	1337	**0**	**0**
										4	614	611	**0**	**1**
Syst. No 17	1000	0	0	0	0	600	400	0	0	**0**	257	242	**6**	**100**
										2	35	27	**0**	**0**
										4	508	508	**0**	**2**
Syst. No 27	0	0	0	0	1000	0	0	1000	0	**0**	370	0	**100**	**100**
										2	524	0	**100**	**100**
										4	346	0	**100**	**100**
Syst. No 31	513	513	0	0	307.5	0	0	0	666.5	**0**	407	284	**30**	**100**
										2	550	543	**1**	**4**
										4	569	572	**-1**	**-2**
Buffer	0	0	0	0	2000	0	0	0	0	**0**	598	0	**100**	**100**
										2	613	0	**100**	**100**
										4	558	0	**100**	**100**

Table 46 Enzymatic stability in whole cells in aqueous organic phase systems determined by HPLC analysis of the D-ABA converted within a 30min reaction. Phase systems were made by the robot. 10.1mg of cells was incubated with 2ml of phase system, made by the robot, for 2 or 4 hours. After that time 10µL solution of DL-ABA (1.33mg) was added to initiate the bioconversion, which was performed for 30min. To determine the initial activity DL-ABA solution was added first, then the cell suspension.

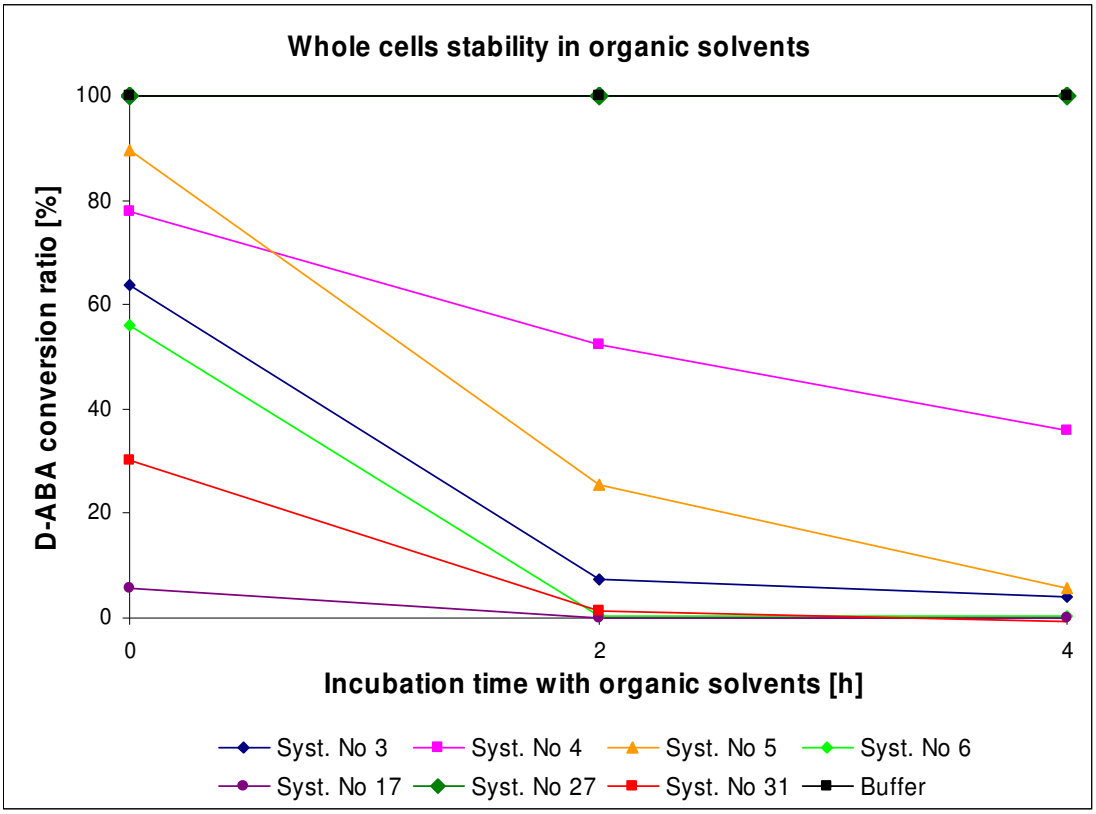


Figure 80 Enzymatic stability of whole cells in aqueous organic phase systems based on Table 46.

6.4.3. Whole cells in ATPS

6.4.3.1. Enzymatic stability of whole cells in ATPS

The main reason for using whole cells in the CCC-bioreactor was the possible higher stability of enzyme. The stability of the enzyme in whole cells was determined in the ATPS, 14/14% PEG1000/potassium phosphate, which was chosen for the CCC-bioreactor. For a control the reaction was performed in 2ml of 50mM sodium phosphate buffer, pH 7.5. To plastic test tubes, 1ml of the upper phase, 1ml of the lower phase and 50µl of aqueous cell suspension (205mg/ml), were placed. The cells were then incubated at room temperature (23°C) in the phase system or in the buffer for 1, 2 or 4 hours on a rotating wheel, which provided a continuous phase mixing. After these times, each time using a new sample, 20µl of the D/L-ABA solution (83.2mg/ml) was added and the reaction was stopped after 15min by derivatising 50µl of a sample for ABA according to the procedure described in section 2.5.1.2d. Results are presented in Figure 81.

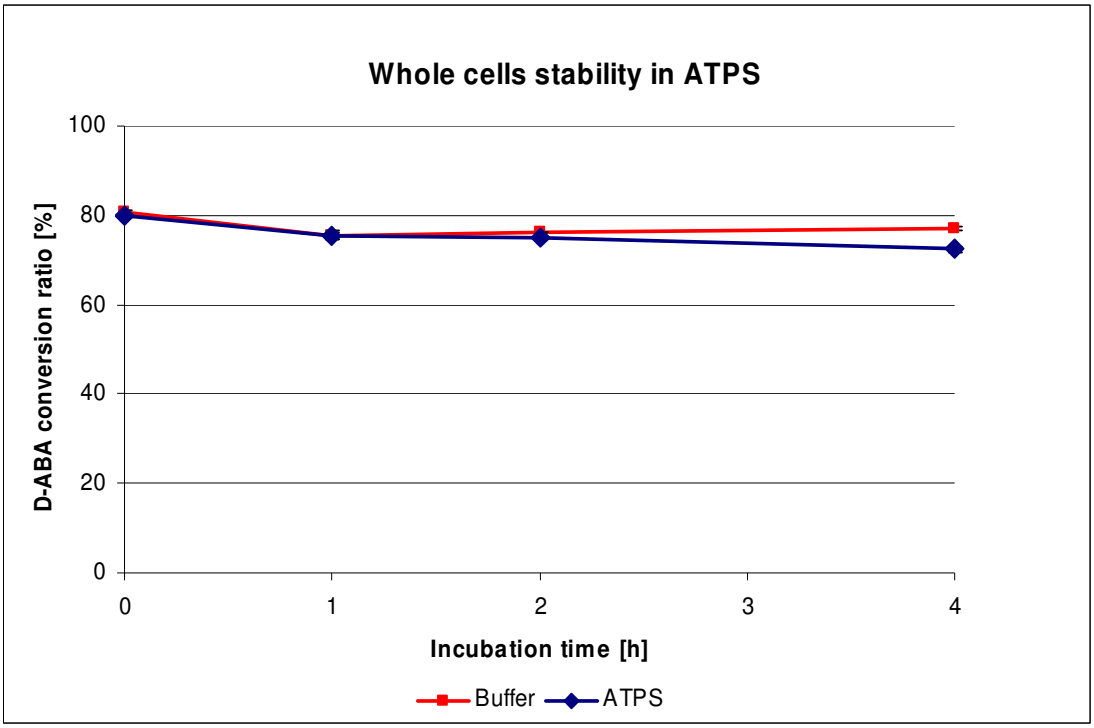


Figure 81 Stability of the enzyme in the whole cells in the ATPS 14/14% PEG1000/potassium phosphate (pH 7.6) and in 50mM sodium phosphate buffer (pH 7.5). The experiment was performed in triplicate. The activity was determined by incubating 10.2mg of *E. coli* with 2ml ATPS or buffer for 1, 2 and 4h. After that time the bioconversion was initiated by adding 20µL solution of DL-ABA (1.6mg) and stopped 15min later by derivation of a sample for ABA. The activity was determined as % of D-ABA converted within 15min reaction.

The stability of the enzyme in whole cells was compared with the stability of the free DAAO (Figure 81). The enzyme in whole cells lost only 5% of its initial activity when incubated for 4h in the buffer and 9% when incubated in the ATPS. The enzyme in whole cells was very stable, both in the buffer and in the ATPS.

Within that time, the free enzyme lost 92% of the initial activity in buffer and 59% in ATPS (Figure 82). That makes the enzyme in whole cells more favourable for the CCC-bioreactor compared to the free enzyme.

In contrast to the experiment presented in section 4.3.7, this new batch of the enzyme was found to be more stable in ATPS than in buffer solution.

Solvent	Time [h]	Whole cells		Free enzyme	
		% of the initial activity	St. error	% of the initial activity	St. error
Buffer	0	100	0.65	100	
	2	95	0.17	29	
	4	95	0.48	8	
ATPS	0	100	0.45	100	1.4
	2	94	0.38	64	0.9
	4	91	0.65	41	0.8

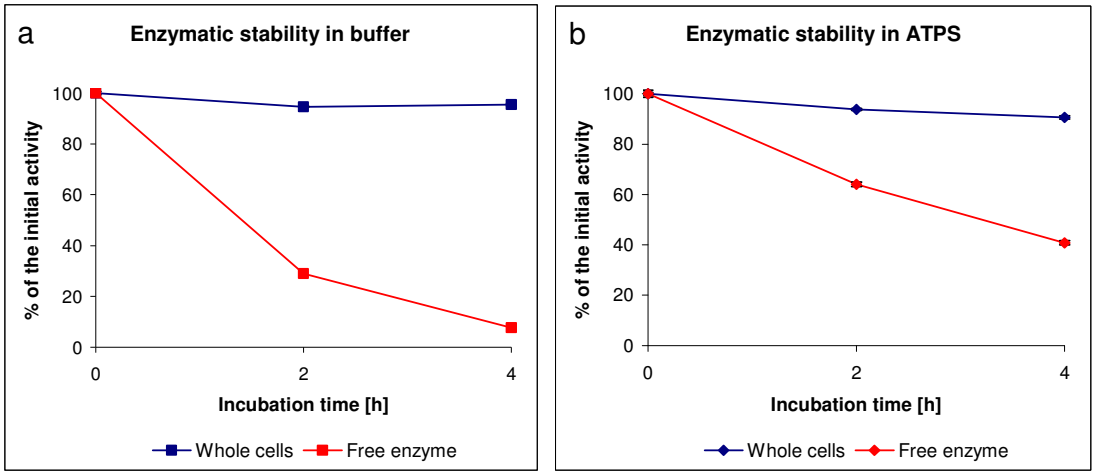


Figure 82 Comparison between the stability of the enzyme in whole cells and the free enzyme. The stability was determined by referring the activity (% converted D-ABA) measured after 2 and 4h to the initial activity which was set at 100%. The stability was determined in 50mM sodium phosphate buffer pH 7.5 (a) and in the ATPS 14/14% potassium phosphate pH 7.6 (b).
To determine the stability of the free enzyme, 20µL was added to 1980µL of the ATPS or buffer. After 2 and 4 hours, 20µL DL-ABA solution was added (3mg) and the reaction was continued for 5min and stopped by derivatising the sample for ABA.

6.4.3.2. Whole cell partitioning in ATPS

Partitioning of the whole cells of *E. coli* was determined in the ATPS based on the enzyme activity. It was assumed that the cells' membrane is integral and enzymatic activity was proportional to the concentration of cells in a sample. Therefore, partitioning of the whole cells could be determined by analysing the enzymatic activity in both phases of the ATPS.

First cell integrity had to be proved. If integral cells are spun down, there should not be any activity in the supernatant. The enzyme activity was determined for the cell suspension (0.202 abs/min, green bar Figure 83a). Next cells were spun down and the enzyme activity was determined for the supernatant (red bar Figure 83a). The activity was 0.024 [abs/min] which means nearly 90% reduction. The enzyme activity did not reduce when the solution of the free enzyme was spun down under the same conditions (Figure 83b), which demonstrates that centrifugation had not inactivated

the enzyme. Therefore the difference in enzymatic activity before and after centrifuging the cell suspension must have been caused by removing integral cells from a solution. This experiment demonstrated that at least 90% of the enzyme is in integral cells, therefore cell concentration can be determined based on the enzyme activity.

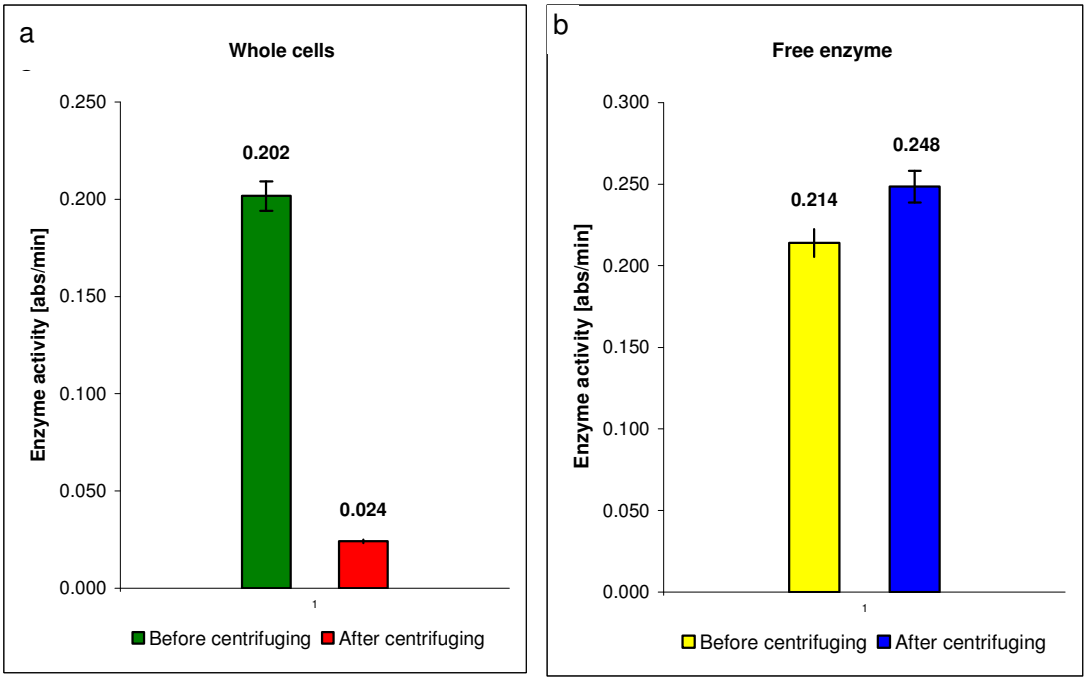


Figure 83 Integrity of *E. coli* with DAAO activity determined by measuring enzymatic activity in a suspension before and after centrifuging. a) Integrity of whole cells. 23.5mg *E. coli* was dissolved in 5ml water. The activity was determined by the colorimetric assay (2.5.2.1). Cells were then spun down on the centrifuge. After this the enzyme activity was determined once again for the supernatant. b) The activity in a solution of the free enzyme, before and after centrifuging.

E. coli was placed into the ATPS previously used with the free enzyme for the successful CCC-bioreactor, (14/14% PEG1000 potassium phosphate). The activity of the enzyme was determined for both phases. Results are presented in Figure 84.

Based on the enzyme activity, the whole cells partition into the lower phase (D-value 0.05), which is not preferable for the CCC-bioreactor. Therefore, another solvent system or a method which alters partitioning, such as cell PEGylation, would need to be found. Both aqueous-organic (section 6.4.2) and ATPS (section 6.4.3) systems were examined together with cell surface PEGylation (section 6.4.3.5).

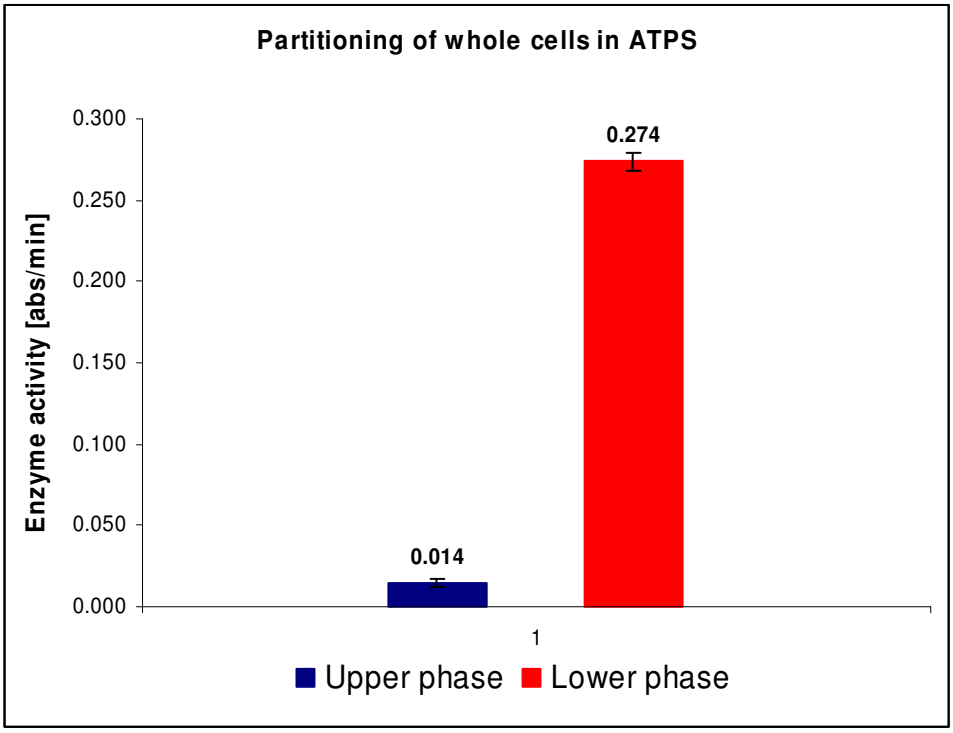


Figure 84 Partitioning of *E. coli* in ATPS (14/14% PEG1000/potassium phosphate) determined by DAAO activity. The experiment was done in triplicate. 14.3mg *E. coli* was placed into 5ml of ATPS, equal amount of the upper and the lower phase. Both phases were analysed for the enzyme activity (2.5.2.1).

6.4.3.3. ATPS selection using a liquid-handling robot

Partitioning of *E. coli* was tested in 98 ATPSs made by means of a liquid-handling robot which had the advantage of making a large number of ATPSs from stock solutions of PEG and various salts in relatively short period of time in comparison to a traditional making of ATPS which would involve an individual weighing of PEG and a salt for each sample.

The distribution ratio of cells was determined by measuring their optical density in the upper and the lower phase of a phase system.

a) Description of a liquid handling robot

A liquid-handling robot (Perkin Elmer Multiprobe II), equipped with 4 tips, and both a syringe and a peristaltic pump was used. The robot transfers the desired volume of a liquid from the source using the syringes. The peristaltic pump is used for flushing the tubing with water, which eliminates any cross contamination. Figure 85 shows the liquid-handling robot.

Figure 85 The liquid-handling robot, Perkin Elmer Multiprobe II

b) Preparing solvents for the robot

A liquid-handling robot is designed to transfer a desired volume of a liquid. It was generally used for making phase systems based on organic solvents and water.[173] The concentration of solvents in those systems is expressed as % v/v, e.g. to make 50/50% butanol/water the same volume of both needs to be taken.

ATPS is made of PEG, salt and water. PEG400 is a liquid at room temperature, PEG1000 is a solid, whereas PEG3350 and all salts selected are powders. Therefore to make them available for the robot, which can transfer only liquids, aqueous solutions of PEG and salts were prepared. Moreover the concentration of PEG and salt in ATPS was expressed as % w/w. For that reason, densities of all stock solutions had to be determined to know the exact weight of PEG and salt in a given volume of transferred stock solution. In Table 47, the results of measuring densities of PEG solutions are presented. The stock solutions of PEG400 and 1000 were 55% w/w concentration. In the case of PEG 3350 and 8000, due to the high viscosity of their aqueous solutions, concentrations of the stock solutions were reduced to 35% and 30% (w/w), respectively.

The density depends only on the PEG concentration, not on its molecular weight (Figure 86).

PEG mol. weight	Concentr. w/w	Empty glass [g] (m_e)	Glass with PEG solution [g] (m_s)		Glass filled with water [g] (m_w)		Specific gravity
400	**55%**	24.4723	51.6752	51.6674	49.3657	49.3636	**1.093**
			51.6702		49.3549		
			51.6568		49.3703		
1000	**55%**	17.2244	44.4743	44.4717	42.0812	42.0988	**1.095**
			44.4664		42.1080		
			44.4745		42.1073		
3350	**35%**	25.1108	51.4047	51.3779	49.8947	49.8747	**1.061**
			51.3742		49.8696		
			51.3547		49.8597		
8000	**30%**	32.6556	85.0487	85.0448	82.4164	82.4230	**1.053**
			85.0451		82.4160		
			85.0406		82.4366		

Table 47 Density of PEG stock solutions used for the robotic ATPS making. The determined density (ρ) was related to the density of water and was calculated according to the equation: $\rho = \frac{m_s - m_e}{m_w - m_e}$; m_s – weight of a volumetric flask totally filled with an analysed solution; m_w – weight of a volumetric flask totally filled with water; m_e – weight of an empty volumetric flask. Volumetric flasks used were of the volume of 25 and 50ml. All weights [g] were determined on an analytical balance (ρ =0.1mg). Solutions and water were brought to 20°C.

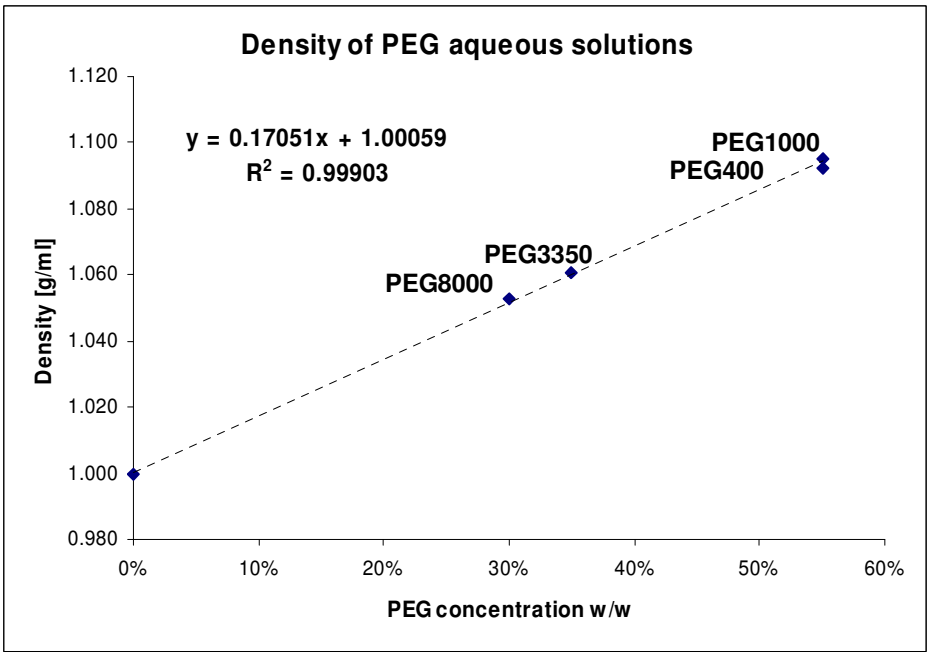


Figure 86 Density of aqueous solutions of PEG. The graph shows a linear relationship between the concentration of PEG solution (%) and the density (g/ml).

Also the density of salt solutions were determined. The concentration of sodium phosphate and sodium sulphate stock solutions were 29% (w/w) due to lower solubility of these salts. Stock solutions of all other salts were made at concentration 40% w/w (Table 48).

Concentr (w/w)	Salt type	Glass no	Salt solution [g] (m_s)		Water [g] (m_e)	Empty [g] (m_w)	Specific gravity
40%	Potassium phosphate	1515	24.3301 24.3303 24.3262	24.3289	20.4985	10.6064	1.387
	Ammonium phosphate	1522	22.8645 22.8595 22.8610	22.8617	20.5244	10.8226	1.241
	Potassium citrate	1522	23.3283 23.3241 23.3252	23.3259	20.5244	10.8226	1.289
	Sodium citrate	1522	23.2223 23.2209 23.2267	23.2233	20.5244	10.8226	1.278
	Ammonium sulphate	1515	22.7746 22.7724 22.773	22.7733	20.4985	10.6064	1.230
29%	Sodium phosphate	1522	23.1986 23.1961 23.1926	23.1958	20.5244	10.8226	1.275
	Sodium sulphate	1522	23.3667 23.3656 23.3634	23.3652	20.5244	10.8226	1.293

Table 48 Density of salts stock solutions used for the robotic ATPS making. The determined density (ρ) was related to the density of water and was calculated according to the equation: $\rho = \frac{m_s - m_e}{m_w - m_e}$;

m_s – weight of a glass pycnometer with a stopper totally filled with an analysed solution,; m_w – weight of a glass pycnometer with a stopper totally filled with water; m_e – weight of an empty a glass pycnometer with a stopper. All weights [g] were determined on an analytical balance (d=0.1mg). Solutions and water were brought to 20°C.

c) Preparing a solvent table for the robot

All ATPSs were made of water and stock solutions of PEG and salts. The volume of added stock solutions of PEG and salts was calculated based on the density presented in Table 47 and Table 48. All calculations were done in Excel (Microsoft Office). The method used for calculating the volume of solutions that the robot used to make ATPS is explained below, based on the example of ATPS 16/16% PEG400/potassium citrate.

In 100g of an ATPS 16/16% there is 16g PEG, 16g of salt and 68g of water. In columns 4-13 (Table 49) calculations are presented to make 4g of ATPS. 4g of ATPS 16/16% PEG400/potassium citrate contains 640mg PEG. That amount of PEG is in 1164mg 55% PEG stock solution (column 5). 1064µL of the stock solution (column 7) needs to be transferred as the density of PEG400 solution (55%) is 1.094g/cm^3 (column 6). The same was calculated for a salt. 4g of the ATPS contains 640mg of potassium citrate which is in 1600mg 40% potassium citrate stock solution (column 9). Therefore 1241µL of the stock solution (column 11) needs to be taken as the density of 40% potassium phosphate is 1.289g/cm^3 (column 10). Column 12 shows a volume of water that needs to be added to make 4g of ATPS. It needs to be noted that the various ATPSs made of different salts and at different concentration would all have the same weight (4g, column 13), but different volumes (column 14), as the final volume of an ATPS depends on the PEG and salt concentration as well as on the salt type. The more concentrated the ATPS, the higher percent of PEG and salt which, being denser then water, affects the total volume (column 14). This complicates the situation. For instance if a fixed sample volume was added to a different volume of ATPS, % concentration of the sample would differ. Moreover the robot might work less effectively if the surfaces of the transferred liquids were at different levels. Therefore to standardise the system, the volume of PEG (column 15), salt (column 16) and water (column 17) was proportionally increased to get a total volume of 4ml in all 98 ATPS. From the calculated volume of water, 100µL was subtracted (column 18) to allow for the addition of a sample usually in an aqueous solution.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
PEG type	**Salt type**	**Concentation**	**PEG**				**Salt**				**Water [g,µl]**	**Total weight [mg]**	**Total volume [µl]**	**PEG volume [µl]**	**Salt volume [µl]**	**Water volume [µl]**	**Sample volume [ul]**	**Total volume [µl]**
			%	**M [mg]**	**Density**	**V [µl]**	**%**	**M [mg]**	**Density**	**V [µl]**								
400	K citrate	16	55	1164	1.094	1064	40	1600	1.289	1241	1236	4000	3541	**1201**	**1402**	**1297**	**100**	4000
400	K citrate	18	55	1309	1.094	1197	40	1800	1.289	1396	891	4000	3484	**1374**	**1603**	**923**	**100**	4000
400	K citrate	20	55	1455	1.094	1330	40	2000	1.289	1552	545	4000	3427	**1552**	**1811**	**537**	**100**	4000
400	K citrate	22	55	1600	1.094	1463	40	2200	1.289	1707	200	4000	3369	**1736**	**2026**	**137**	**100**	4000
400	KPO4	16	55	1164	1.094	1064	40	1600	1.387	1154	1236	4000	3454	**1232**	**1336**	**1332**	**100**	4000
400	KPO4	18	55	1309	1.094	1197	40	1800	1.387	1298	891	4000	3385	**1414**	**1533**	**953**	**100**	4000
400	KPO4	20	55	1455	1.094	1330	40	2000	1.387	1442	545	4000	3317	**1603**	**1739**	**558**	**100**	4000
400	KPO4	22	55	1600	1.094	1463	40	2200	1.387	1586	200	4000	3249	**1801**	**1953**	**146**	**100**	4000
400	Na citrate	16	55	1164	1.094	1064	40	1600	1.278	1252	1236	4000	3552	**1198**	**1410**	**1292**	**100**	4000
400	Na citrate	18	55	1309	1.094	1197	40	1800	1.278	1408	891	4000	3496	**1369**	**1612**	**919**	**100**	4000
400	Na citrate	20	55	1455	1.094	1330	40	2000	1.278	1565	545	4000	3440	**1546**	**1820**	**534**	**100**	4000
400	Na citrate	22	55	1600	1.094	1463	40	2200	1.278	1721	200	4000	3384	**1729**	**2035**	**136**	**100**	4000
400	NaPO4	16	55	1164	1.094	1064	29	2207	1.275	1731	629	4000	3424	**1243**	**2022**	**635**	**100**	4000
400	NaPO4	18	55	1309	1.094	1197	29	2483	1.275	1947	208	4000	3352	**1428**	**2324**	**148**	**100**	4000
400	NaSO4	16	55	1164	1.094	1064	29	2207	1.293	1707	629	4000	3400	**1251**	**2008**	**641**	**100**	4000
400	NaSO4	18	55	1309	1.094	1197	29	2483	1.293	1920	208	4000	3325	**1440**	**2310**	**150**	**100**	4000

Table 49. Part of the calculations made to program the liquid-handling robot to make ATPS. The whole table is in the appendix on p217 to 219. Column 1) molecular weight of PEG used ; Column 2) salt type; column 3) concentration of both PEG and a salt in ATPS; column 4) concentration of PEG stock solution used [% w/w]; column 5) amount [mg] of stock solution of PEG to make 4g of ATPS; column 6) density of PEG stock solution [g/cm^3]; column 7) volume [µl] of stock solution of PEG to make 4g ATPS; 8) concentration of salt stock solution used [% w/w]; column 9) amount [mg] of stock solution of salt to make 4g of ATPS; column 10) density of salt stock solution [g/cm^3]; column 11) volume [µl] of stock solution of salt to make 4g ATPS; 12) volume of water to make 4g ATPS; column 13) total weight [µg] of ATPS; column 14) total volume [µl] of 4g ATPS; column 15) volume [µl] of PEG stock solution to make 4ml ATPS; column 16) volume [µl] of salt stock solution to make 4ml ATPS; column 17) volume of water to make 4ml ATPS; column 18) volume of a sample; column 19) total volume

d) Determining the distribution ratio of *E. coli* in ATPS

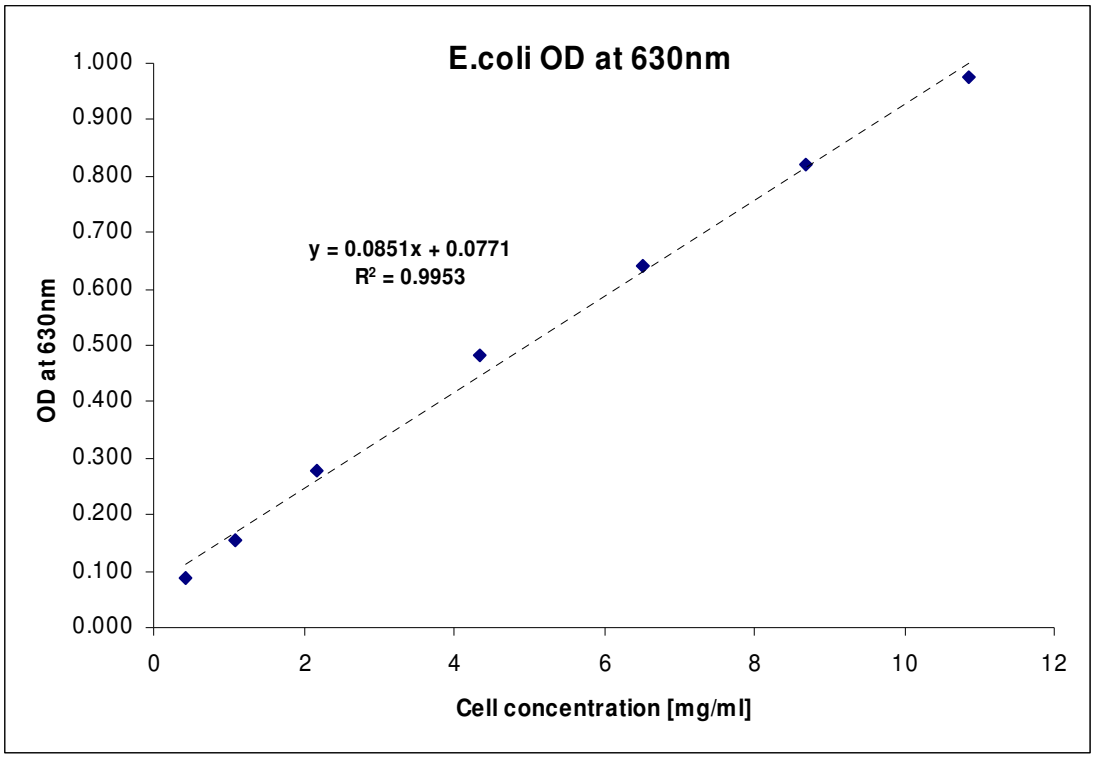


Figure 87 The correlation between the *E. coli* cell concentration and OD measured at 630nm on the plate reader. 300µl of *E. coli* suspension was placed into a well of 96-well plate. The OD was measured at 630nm.

The robot made 98 ATPSs. To each of them, 100µl of cell solution (8.0mg cells) was added, so the cell concentration in each tube was 2.0mg/ml. The volume ratio of the two phases for most systems was close to 1. Therefore, if all cells were distributed in one phase their concentration (4mg/ml) would still be within the linear detection range (Figure 87). After manually shaking, the samples were left for 30min to allow the two phases to separate. 300µl of each phase was separately placed by the robot into the well of a 96-well plate. Then the optical density at 630nm (OD_{630}) was determined. This was the closest available wavelength to 600nm, the standard wavelength used to determine the optical density of cells.

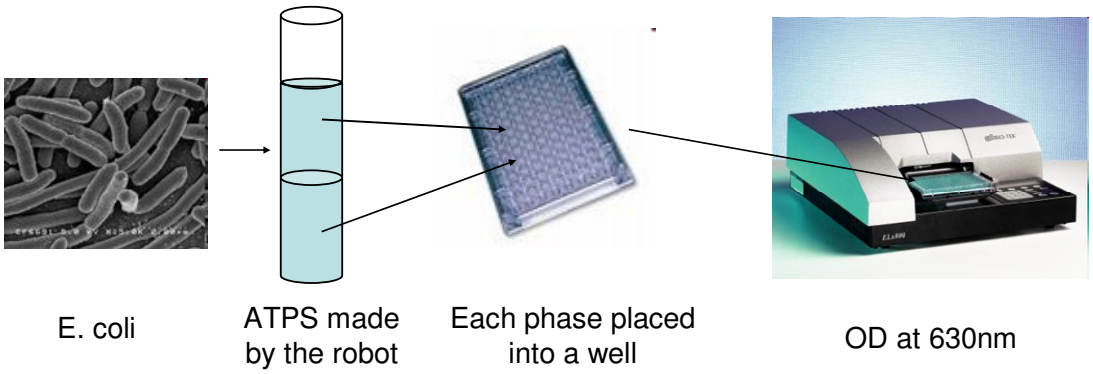


Figure 88. Determination of *E. coli* partitioning in ATPS made by the liquid-handling robot. Suspension of *E. coli* cells were added to ATPS made by the robot, which then transferred separately the upper and the lower phase of the phase system to 96-well plate. The OD_{630} was determined by the plate reader.

The distribution ratio was determined for each ATPS by dividing OD_{630} obtained for the upper phase by OD_{630} obtained for the lower phase.

The expected total OD_{630} was between 0.5 [abs], if cells were equally distributed between the two phases and 1.0 [abs] if cells totally partitioned into one phase. However, the cells frequently partitioned into the interface area and in that case the sum of OD_{630} for both phases was much lower than 0.5 [abs]. From the results it was possible to select 8 ATPSs where the distribution ratios were measurably over 10 (Table 50). A high distribution ratio is desirable as it means the cells partition almost entirely to the stationary phase of the CCC-bioreactor. Where these samples show a very high SD, this indicates a heterogeneous distribution of cells in the phase systems as it was calculated from 5 OD_{630} readings of the same samples.

No	PEG molecular weight	Salt type	Concentr [% w/w]	D-value of whole cells	SD
1	400	NaSO4	18	**60**	**48**
2	400	NaSO4	20	**22**	**22**
3	1000	NaPO4	16	**12**	**7**
4	1000	NaPO4	18	**231**	**202**
5	1000	NaSO4	14	**256**	**412**
6	1000	NaSO4	16	**17**	**3**
7	1000	NH4SO4	16	**114**	**13**
8	1000	NH4SO4	18	**648**	**514**

Table 50 Selected phase systems where the whole cells partition mainly to the top, PEG-rich phase, giving a D-value over 10. The D-value was determined by dividing OD_{630} for an upper phase by OD_{630} for a lower phase. OD_{630} for each phase was determined 5 times.

The two ATPSs made of ammonium sulphate (NH_4SO_4) were rejected as this salt makes the ABA analysis difficult (2.5.1.2a). To achieve satisfactory cell partitioning, ATPS based on PEG 400 needs to be made at high concentration which make the ATPS expensive. Therefore the best phase systems seem to be an ATPS made of PEG1000 and either sodium sulphate or sodium phosphate.

There was a slight inconsistency between the experiments presented in Table 50 and Tables 50 and 51. By contrast to the experiment presented in Table 50, when all ATPS were made by the robot, this time ATPS were made by weighing and mixing all components (PEG, salt and water) separately, which is considered to be more accurate. In ATPSs made of PEG1000 and sodium sulphate, only at the concentration of 14/14% did the cells partition into the upper PEG-rich phase. At higher ATPS concentrations, the cells partitioned to the interface area (Table 51), which can indicate instability of cells in this phase system. Only a visual observation was done as most of the material was visible in the interface area.

PEG1000/sodium sulphate	
ATPS concentration [w/w%]	Cells partitioning
14	upper phase
16	interface
18	interface
20	interface

Table 51 Whole cell partitioning in ATPS made of PEG1000 and sodium sulphate. 9.2mg of cells were placed into 4ml of ATPS. Due to the cloudiness of the cell suspension, it was possible to observe where the majority of the cells lay.

In case of the ATPS made of PEG 1000 and sodium phosphate, cell partitioning strongly depends on the ATPS concentration (

Table 52). The higher the ATPS concentration, the more cells partition to the upper, PEG-rich phase. The D-value was determined based on DAAO activity in whole cells.

ATPS concentration [w/w%]	**D-value**	**St. error**
12.5	0.1	0.01
14	2.6	0.13
16	8.3	0.24
18	10.7	0.32

Table 52 Partitioning of whole cells of *E. coli* in ATPS made of PEG1000 and sodium phosphate determined by comparing DAAO activity in the colorimetric enzyme assay. Standard error calculated for 3 replications.

Based on the cell partitioning, the phase system for the CCC-bioreactor was chosen as 16/16% PEG1000/sodium phosphate. The concentration 14/14% did not provide a sufficiently high D-value of cells, whereas 18/18% was rejected because of the risk of salt precipitation.

6.4.3.4. Calculation of the whole cell concentration in SP required for the CCC-bioreactor

The concentration of whole cells to be used in the stationary phase was calculated, based on the enzymatic activity of these cells compared to the activity of the free enzyme used for the CCC-bioreactor (5.2.2). According to the colorimetric enzyme assay (2.5.2.1), the enzymatic activity of the 2% free enzyme solution, obtained by dissolving 2ml of the enzyme in 100ml of stationary phase, was 0.6 abs/min. Based on the enzymatic activity of whole cells, to obtain this activity in 100ml of the stationary phase, 1.1g of whole cells needed to be used.

Therefore, 1.17g of *E. coli* was suspended in 100ml of the upper phase of 16/16% PEG1000/sodium phosphate. First, cells were mixed with 2ml of water, then added to the upper phase and mixed by an electromagnetic stirrer for 10min. Surprisingly the pump blocked when the stationary phase containing the cells was pumped onto the CCC column. It was noticed that cells in the stationary phase were flocculating and then sedimenting. The flocculated cells also caused the CCC coil to block. For that reason the experiment was stopped and the CCC-bioreactor was not performed using whole cells.

It needs to be mentioned that in any of the experiments presented in the previous section, no flocculation of cells in ATPS was observed. This might be explained by a different type of mixing applied to the partitioning studies. The suspension of cells was placed in ATPS and mixed by manually inversing the test tube several times. This mixing was effective enough to the distribute cells evenly in a phase system but not so vigorous as to initiate flocculation of cells, as accrued when more powerful electromagnetic stirring was applied.

6.4.3.5. Effect of Polyethylene Glycol Monostearate on *E. coli* sedimentation in ATPS

A method that prevents *E. coli* from flocculation in ATPS was required. Therefore, the use of Polyethylene Glycol Monostearate to prevent flocculation was investigated. Polyethylene Glycol Monostearate (n=10), palmitate and stearate mixture (PEG-MS, CAS number 9004-99-3), was purchased from TCI America.

E. coli cells premixed with PEG-MS were placed into the upper phase of ATPS. The OD_{600} was determined at time 0 and 4h using a spectrophotometer (Shimadzu Deutschland GmbH). The beam of the light passed through the side of a cuvete. Therefore, the difference in OD_{600} was assumed to be caused by cell sedimentation. In the experiment, four factors were analysed, namely salt type, ATPS concentration, cell concentration and PEG-MS concentration. To reduce the total number of conditions and experiments to be investigated, a design of experiment software (Design-Expert®, Stat-Ease, Inc) was used.

Two types of ATPS were analysed, PEG1000/sodium sulphate and PEG1000/sodium phosphate at the concentration of 14/14, 16/16 and 18/18%. Cell concentration in ATPS varied from 2 to 20mg/ml and PEG-MS from 0 to 5mg/ml. For those conditions, the Design Expert program generated 18 variations. These were then tested experimentally as follows. To each plastic cuvete was placed the cell suspension (2.1g of cells mixed with 1ml water), PEG-MS solution (243mg/ml) and water in the amounts given in the last 3 columns in Table 53.

Std	ATPS concentr. [% w/w]	Cells [mg/ml]	PEG-MS [mg/ml]	Salt type	ATPS [ml]	Cells [g]	PEG-MS [µL]	Water [µL]
1	14	2	0	Sulphate	4.0	8	0	192
2	18	20	0	Sulphate	4.0	80	0	120
3	14	2	5	Sulphate	4.0	8	103	89
4	18	2	5	Sulphate	4.0	8	103	89
5	14	20	5	Sulphate	4.0	80	103	17
6	18	20	5	Sulphate	4.0	80	103	17
7	14	2	0	Phosphate	4.0	8	0	192
8	18	2	0	Phosphate	4.0	8	0	192
9	14	20	0	Phosphate	4.0	80	0	120
10	18	20	0	Phosphate	4.0	80	0	120
11	18	2	5	Phosphate	4.0	80	103	89
12	14	20	5	Phosphate	4.0	44	103	17
13	16	11	2.5	Sulphate	4.0	44	51	105
14	16	11	2.5	Phosphate	4.0	44	51	105
15	16	11	2.5	Sulphate	4.0	44	51	105
16	16	11	2.5	Phosphate	4.0	44	51	105
17	16	11	2.5	Sulphate	4.0	44	51	105
18	16	11	2.5	Phosphate	4.0	44	51	105

Table 53 Composition of samples used to determine effects of ATPS concentration, salt type, cells concentration and presence of PEG-MS on *E. coli* sedimentation in the upper phase of ATPS.

After 1 hour, 4ml of upper phase of ATPS was added to each cuvete and the absorbance measured at 600nm using a spectrophotometer. The samples were then left for 4 hours to allow the cells to settle down. After that time, the absorbance was measured again. The sedimentation rate (S) after 4h was calculated according to Equation 13.

$$S = 100 - \frac{OD_{t=4}}{OD_{t=0}} \cdot 100\,;\,[\%] \qquad \text{Equation 13}$$

where $OD_{t=0}$ is the optical density measured at 600nm at the time 0, $OD_{t=4}$ is the optical density measured at 600nm after 4 hours. Results are shown in Table 54.

Std	ATPS concentr. [w/w %]	Cells [mg/ml]	PEG-pal [mg/ml]	Salt type	Absorbance		Sedimentation after 4h [%]
					t=0h	t=4h	
1	14	2	0	Sulphate	0.625	0.690	**-10**
2	18	20	0	Sulphate	2.686	0.900	**66**
3	14	2	5	Sulphate	2.159	0.628	**71**
4	18	2	5	Sulphate	1.406	0.260	**82**
5	14	20	5	Sulphate	3.163	1.250	**60**
6	18	20	5	Sulphate	2.950	1.624	**45**
7	14	2	0	Phosphate	0.656	0.401	**39**
8	18	2	0	Phosphate	0.580	0.479	**17**
9	14	20	0	Phosphate	2.804	0.823	**71**
10	18	20	0	Phosphate	2.640	0.567	**79**
11	18	2	5	Phosphate	2.057	0.628	**69**
12	14	20	5	Phosphate	3.096	1.670	**46**
13	16	11	2.5	Sulphate	2.700	1.273	**53**
14	16	11	2.5	Phosphate	2.600	1.043	**60**
15	16	11	2.5	Sulphate	2.421	1.578	**35**
16	16	11	2.5	Phosphate	2.486	1.084	**56**
17	16	11	2.5	Sulphate	2.342	1.302	**44**
18	16	11	2.5	Phosphate	2.520	1.070	**58**
19	0	2	0	Water	1.100	1.107	**-1**
20	0	20	0	Water	3.015	3.003	**0**

Table 54. Sedimentation calculated for tested samples by comparing OD600 measured immediately after placing cells treated with PEG-MS to ATPS and after 4hours.

Calculated values of sedimentation (S) shown in Table 54, as well as analysed parameters (ATPS concentration, cell concentration, PEG-MS concentration and salt type), were typed to the Design Expert table to perform statistical analysis.

Table 55 shows analysis of variance (ANOVA) generated by the Design Expert. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case B, C, D, BC, CD, ABC are significant model terms. Based on this information cell sedimentation depends on cell concentration, PEG-MS concentration and the type of salt used for ATPS and is independent of ATPS concentration.

In needs to be noted that based on Table 51 (section 6.4.3.3d), concentrated ATPS caused the flocculation of *E. coli* as they partitioned to the interface area. However, that experiment was performed in the absence of polyethylene glycol monostearate.

ANOVA for Selected Factorial Model
Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	7121.429	9	791.2698	14.17567	0.0010
A	3.375	1	3.375	0.060463	0.8128
B	570.375	1	570.375	10.21832	0.0151
C	979.4579	1	979.4579	17.54707	0.0041
D	320.6429	1	320.6429	5.744344	0.0477
AB	15.85786	1	15.85786	0.284095	0.6105
AC	1.041667	1	1.041667	0.018662	0.8952
BC	4030.042	1	4030.042	72.19854	< 0.0001
CD	780.125	1	780.125	13.97601	0.0073
ABC	392	1	392	7.022714	0.0329

Table 55 ANOVA test for the analysed samples generated by Design Expert.

Figure 89, generated by Design Expert, presents a correlation between PEG-MS concentration [mg/ml] and cell sedimentation after 4h [%] at the cell concentration of 11mg/ml for two ATPS, PEG1000/sodium phosphate and PEG1000/sodium sulphate, both at the concentration of 16/16% (w/w).

The simulation was performed for a cell concentration of 11mg/ml as that concentration had previously been chosen for the CCC-bioreactor based on the enzyme activity (7.3.5.). The ATPS concentration was 16/16%. As mentioned above, cell sedimentation is independent of that factor, therefore the simulation could be performed for any ATPS concentration within the range of 14-18%.

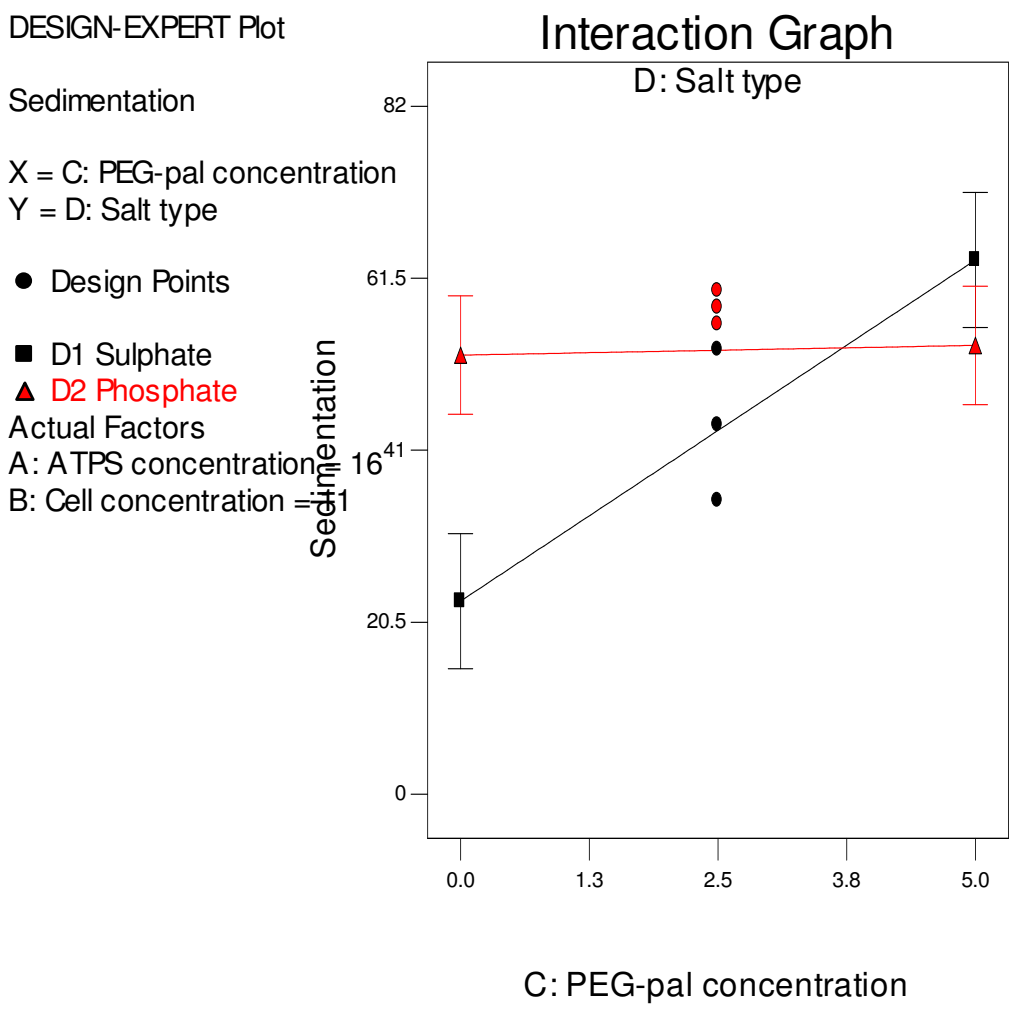


Figure 89 Effect of cells and PEG-MS (PEG-pal) concentration in the upper phase of 16/16% PEG1000/sodium sulphate and 16/16% PEG1000/sodium phosphate on cell sedimentation determined by Design Expert program.

In Figure 89 there are two lines showing the sedimentation of *E. coli* cells in PEG1000/sodium sulphate (black line) and PEG1000/sodium phosphate (red line). At the cell concentration of 11mg/ml, the sedimentation is independent of the presence of PEG-MS in the upper phase of PEG1000/sodium phosphate (red line). After 4 hours about 50% cells sedimented regardless of PEG-MS concentration. The presence of PEG-MS in PEG1000/sodium sulphate increase cell sedimentation from 25% where no PEG-MS was used to over 60% at the PEG-MS concentration of 5mg/ml.

Since the presence of PEG-MS does not prevent *E. coli* from sedimentation when used at the concentration of 11mg/ml, this method is not adequate for the project.

6.5. Conclusion

As expected, the enzyme DAAO is much more stable when used in whole cells, rather than as a free, isolated form. Incubation for 4h in ATPS lost only 5% of its initial activity (6.4.3.). An unexpected challenge was the partitioning of the *E. coli* cells to the desired phase of a phase system. Using a robotic phase system selection approach the ATPS, 16/16% (w/w) PEG1000/sodium phosphate, was selected due to a preferable partitioning of the cells to the top, PEG-rich phase. Nevertheless under these conditions, cells of *E. coli* have a tendency to flocculate and precipitate out of the phase system, which greatly reduces their effectiveness in the CCC-bioreactor. A PEG-derivative, polyethylene glycol monostearate did not stop the cells from flocculation in ATPS.

None of the tested organic solvent systems (6.4.2.) could provide both enzyme stability and preferable partitioning of ABA. Therefore this type of a solvent system was rejected as a phase system for the CCC-bioreactor with whole cells of *E. coli*.

MAO in the whole cells of *E. coli* was not investigated due to a very low enzymatic activity (6.3) of the obtained cells as confirmed by applying the isolated form of the enzyme (6.3.4). Moreover, as the enzyme was in the whole cells of *E. coli,* the problem with flocculation would be likely to arise as in the previous example.

Despite the high enzymatic stability in whole cells, they were not used for the CCC-bioreactor because of their flocculation in the most favourable phase systems.

Further work is required to identify an agent that would stop cells from flocculation in ATPS. Another possibility is either finding a suitable phase system or a different type of cell which would not have a tendency to flocculating.

7. CONCLUSION

The presented work shows the first successfully created CCC-bioreactor which, at the same time, can provide both a total conversion of the injected substrate and a complete separation of products of the reaction.

In the literature there are a few examples of the use of countercurrent chromatography for a combined bioconversion and separation. However, a successfully created CCC-bioreactor capable of both a total conversion of an injected substrate and a complete separation of the reaction products, has not been described. For instance, Bousquet *et al*[138] achieved only 80% of enantiomeric purity when countercurrent chromatography was applied for the enantioselective hydrolysis of 2-cyano cyclopropyl-1,1 dicarboxylic acid dimethylesters with a bacterial esterase present in the aqueous stationary phase. The whole process took 8 hours. In turn, Hollander and co-workers[141,142] investigating a bioreactor based on CPC for the enantioseparation of amino acids did not resolve peaks completely. Moreover, the authors reported a small amount of unconverted substrate eluting from the column, which would indicate incomplete deracemisation of the injected substrate. Also Armstrong *et al*[140] did not achieve a total conversion of the substrate in CPC used as a combined bioreactor/separator. The enzyme α-chymotrypsin, used to perform the enantioseparation of DL-tryptophan methyl ester, was found to be unstable in the aqueous - organic phase system applied in the project.

In contrast to the works of other research groups who tried to apply CCC as a combined bioreactor and separator, in this research project not only the total bioconversion of the injected substrate and resolution of the peaks were achieved, but comprehensive optimisations of the process as well as the phase system selection were presented.

The use of a countercurrent centrifuge bioreactor/separator for manufacture of enantiopure small molecules was demonstrated on a model reaction of deracemisation of DL-amino butyric acid using a Brunel-made Milli CCC machine. The technique allowed isolation of L-ABA of an enantiomeric purity >99% ee, free of KBA, the product of the enzymatic conversion, as well as the enzyme which catalysed the reaction.

All the aims and objectives listed in Chapter 1 have been met. Analytical procedures which allowed ABA, KBA and enzyme activity to be analysed were either developed or adopted to the presence to ATPS. A significant challenge was the development of analytical methods which were not affected by the presence of the phase systems. Since both ABA and KBA are small molecules, HPLC was selected as an analytical method. GC was not used due to incompatibility of this technique with samples containing salts at high concentration, such as ATPS.

Four HPLC methods were investigated to analyse ABA and KBA. Only a HILIC column enabled simultaneous analysis of the two compounds. Despite this great advantage and the fact that there was no requirement for a sample to be derivatised, this method had the downside of low sensitivity, lack of differentiation between the two chiral forms of ABA and incompatibility with some phase systems. Therefore, whenever a concentration of both ABA and KBA had to be determined, each sample was analysed twice, using separate methods for ABA and KBA. Due to a higher sensitivity, samples were usually analysed for KBA on a C18 column, rather than on an anion exchange column. Moreover, the C18 HPLC columns lasted much longer than the anion exchange type, which reduced the cost of analyses. The drawback of this method was the necessity of derivatisation of each sample. To analyse ABA, derivatisation with ortho-phthalaldehyde was used. Replacing the reducing agent, 2-mercaptoethanol, by N-isobutyryl-L-cysteine allowed the two chiral forms of ABA to be separated on a standard C18 column. Due to the presence of ammonium cations, the procedure of ABA derivatisation was not compatible with phase systems made of ammonium sulphate, the salt which was initially selected for the reason of requirement for KBA reduction in the next step. This was overcome by extracting ABA with methanol, which does not solubilise ammonium sulphate.

An analytical method was also needed to analyse partitioning of the enzyme in a phase system. This could be done either by measuring enzyme activity or concentration of protein. Most of the available protein concentration assays were either not sensitive enough (Biuret assay, absorbance measurement at 280nm) or not compatible with ATPS. This was mainly a result of either a high concentration of phosphate salts, which having a very strong buffering effect would interfere with such assays as Lowry, Bradford and BCA, or the presence of ammonium cations, which would react with ortho-phthalaldehyde. Therefore partitioning of the enzyme was

determined by measuring its enzymatic activity. Moreover, it was more important to know how much enzymatic activity was in each phase, rather then enzyme molecules, which could be inactivated.

Phase system selection is a big part of each CCC separation. This can take hours, days or even months. In this project, selection of an appropriate phase system was even more challenging due to additional necessary criteria. For effective bioreactor/separator action, a high partitioning of the biocatalyst to the stationary phase was required in order to retain the biocatalyst in the coil, with differing partitions of substrates and products between the stationary phase (SP) and mobile phase (MP) so that these could be separated. Selecting an appropriate phase system was proceeded by testing the whole range of two-phase systems such as aqueous two-phase systems (ATPS), aqueous–ionic liquid phase systems and aqueous organic phase systems. ATPS is used for a preparative extraction of such enzymes as α-amylase, β-xylosidase, endoglucanase, glucoamylase, α-1-antytripsin, α-galactosidase. Unexpectedly, the enzyme used in this project turned out to be unstable in most of the tested phase systems, including ATPS which was well reported to be effective in preserving enzyme activity. Therefore, one of the most important parameters in the selecting a phase system was enzyme stability. Due to promising initial results as well the recommendations found in the literature, the research concentrated on ATPS. The enzyme stability, as well as the distribution ratios of DL-ABA, KBA and DAAO were measured in a range of phases with polyethylene glycols (PEGs) of different molecular weights, different salts, and different compositions of PEG and salt. There are many different approaches for phase system selection for CCC. This can involve, for instance, trial and error methods, testing a range of solvent systems, or testing phase systems reported in the literature for separation of similar compounds. In this research a systematic approach was applied and the effect of only one factor at a time was tested on enzyme activity. Later in the project, when the partitioning of cells in ATPS was tested, more sophisticated methods involving the liquid-handling robot and DoE software were used. The enzyme was found to be most stable in phase systems made of high molecular weight PEG, such as 3350 at a relatively low concentration. However, under such conditions the enzyme did not partition into the preferred top, PEG-rich phase. It was discovered that this could be overcome by applying the reaction of PEGylation to the enzyme. However, due to the extra cost and inconvenience, this approach was not taken further. Instead ATPS made

of PEG1000 at the concentration of 14/14% was applied, which did not require the use of PEGylated enzyme. A system of 14% w/w PEG 1000 and 14% w/w potassium phosphate, pH 7.6, gave the best combination of distributions ratios ($C_{PEG\ phase}/C_{salt\ phase} = C_{SP}/C_{MP}$) for ABA, KBA and biocatalyst (DAAO) of 0.6, 2.4 and 19.6 respectively.

Compared to aqueous-aqueous phase systems, aqueous-organic phase systems are known to give more efficient mixing between phases and better stationary phase retention in CCC. This results in much higher resolution between separated compounds. Since in the literature there are reported examples of successfully performed bioconversions in the presence of organic solvents, even under totally anhydrous conditions, a short study was done where a number of aqueous-organic phase systems were examined. Initially, some aqueous-organic phase systems showed promise, with high enzyme activity. However, it was discovered that in these systems the enzyme remained in the aqueous phase. Due to a large polarity difference between the phases, ABA and KBA also partitioned totally into the aqueous phase and would therefore never elute from CCC if this phase was used as the stationary phase. In all phase systems where the enzyme contacted organic solvents, a loss of activity was observed.

Also ionic liquid (IL) two-phase systems were also reviewed, due to their potential as a phase system reported in the literature,. For instance it has been reported that four different proteins: cytochrome C, myoglobin, ovoalbumin and haemoglobin all partitioned totally into the IL phase in [BMIM][Cl]/K_2PO_4 phase system. This was desired as the enzyme would be retained in IL-phase, the stationary phase in the CCC-bioreactor. Furthermore, DAAO was reported to be relatively stable in two water immiscible IL, [BMIM][Tf2N] and [BMIM][PF6], where it maintained over 80% of the initial activity after 24h incubation at 30°C. Moreover, a very low vapour pressure, makes ILs nonvolatile solvents. Therefore, the composition of phase systems consisting of IL does not change. This allows reusing of a phase system, which would reduce the overall cost of the process. Unfortunately, this enzyme did not maintain activity in most of the tested ILs. Therefore, these phase systems were not used as a phase system in the CCC-bioreactor.

To achieve a complete deracemisation of ABA, unwanted D-ABA had to be totally converted to its keto analog, KBA, otherwise it would contaminate the product L-ABA by coeluting with it. Therefore, CCC operating conditions, such as substrate concentration, biocatalyst concentration, the mobile phase flow rate (residence time in

the CCC coil), temperature and rotational speed, were optimised. Effects of these conditions on D-ABA conversion were predicted and measured. Some surprises were encountered. For example, in contrast to most enzymatic reactions, a higher bioconversion was achieved at a lower temperature. Operational modes (single flow and Multiple Dual-Mode) and types of mixing (cascade and wave-like) were also optimised. Between the two types of mixing, wave-like mixing turned out to be more efficient, as being less vigorous it broadened the ABA plug, extending the contact with the enzyme and giving better conversion.

For the optimum run conditions of the CCC-bioreactor, a plug of the racemate of ABA in the amount of 8mg was injected with the mobile phase pumped at the flow rate of 0.5ml/min with the CCC column (45ml) rotated at the rotational speed 1800rpm. The concentration of the enzyme D-amino acid oxidase in the stationary phase was $0.4 g \cdot l^{-1}$ and the temperature of the CCC coil 20°C. Multiple Dual-Mode was applied to achieve a complete separation of L-ABA from KBA. The process took 300min, however L-ABA finished eluting 160 min after the injection, so if the intention was to collect only the pure, single enantiomer, the run could be stopped after this time. L-ABA of an enantiomeric purity >99% ee was free of KBA, the product of the enzymatic conversion.

The biggest advantage of applying the CCC-bioreactor is the isolation of enantiomerically pure L-ABA in a single step, as both the bioconversion of unwanted D-ABA and the separation of the produced KBA occurred at the same time. Moreover, not only did the isolated L-ABA elute free of KBA, but also free of the enzyme, which offers the potential to use the CCC-bioreactor in a continuous mode. However, a subsequent substrate injection was not possible due to the unpredicted instability of the enzyme in the phase system.

Despite the advantages of the CCC-bioreactor over current commercial technology, such as reduced equipment footprint, faster purification and possible continuous operation mode, its drawbacks arising from the enzyme instability and excessive optimisation time, as well as a low throughput, reduces its commercial appeal. However, this technique may have a commercial potential in the isolation of high valuable enantiomers such as L-amino acids. In order to decrease enzyme consumption, which is the factor that most affects the cost of the whole process, multiple injections should be applied. This would allow the same portion of enzyme to catalyse many batches of substrate loaded into the CCC-bioreactor. However, this

would require use of a much more stable enzyme. Engineering the enzyme to achieve extended stability in phase systems might be the key to make the process more economical. As mentioned, a further limitation of the process comes from the low substrate loading, which makes the ratio of obtained product to enzyme used in the process unprofitable. However, this can be overcome by improving the enzyme activity, or perhaps applying other reactions such as enantioselective deamination.

Future work should include investigation into improving the enzyme stability and enhancing the substrate loading. Investigations into the use of the biocatalyst in the whole cells of *E. coli* showed the potential to overcome the problem with the enzyme instability. Eventually, due to flocculation in ATPS, *E. coli* cells were not used in the CCC-bioreactor. However, there are other types of host cells, such as *Pichia pastoris,* available with the activity of D-amino acid oxidase, and these should be considered. In addition, other phase systems can be examined to find ones that give little flocculation. For example PEG-dextran phase systems prepared in isotonic buffers have been widely used for cell separations. This would require optimisation of the distribution ratios of all components, and then optimisation of the operating conditions. However, further complications such as a difficulty of product extraction from the dextran-phase, may arise from applying this kind of a phase system.

A very low substrate loading is a factor which, at this time, makes the process uneconomical. This might have been caused by the saturation of the FAD-dependent enzyme, which in the absence on oxygen could not regenerate, or the elution of FAD together with the mobile phase. Additional research should be performed to investigate these possibilities and develop ways of overcoming the limitations, if necessary. Alternatively, enzymes catalysing other reaction types, such as enantioselective, cofactor-independent hydrolase should be investigated.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
No	**PEG type**	**Salt type**	**Concentration**	**PEG**				**Salt**				**Water [g,µl]**	**Total weight [mg]**	**Total volume [µl]**	**PEG volume [µl]**	**Salt volume [µl]**	**Water volume [µl]**	**Sample volume [ul]**	**Total volume [µl]**
				%	**M [mg]**	**Density**	**V [µl]**	**%**	**M [mg]**	**Density**	**V [µl]**								
1	400	K citrate	16	55	1164	1.094	1064	40	1600	1.289	1241	1236	4000	3541	**1201**	**1402**	**1297**	**100**	4000
2	400	K citrate	18	55	1309	1.094	1197	40	1800	1.289	1396	891	4000	3484	**1374**	**1603**	**923**	**100**	4000
3	400	K citrate	20	55	1455	1.094	1330	40	2000	1.289	1552	545	4000	3427	**1552**	**1811**	**537**	**100**	4000
4	400	K citrate	22	55	1600	1.094	1463	40	2200	1.289	1707	200	4000	3369	**1736**	**2026**	**137**	**100**	4000
5	400	KPO4	16	55	1164	1.094	1064	40	1600	1.387	1154	1236	4000	3454	**1232**	**1336**	**1332**	**100**	4000
6	400	KPO4	18	55	1309	1.094	1197	40	1800	1.387	1298	891	4000	3385	**1414**	**1533**	**953**	**100**	4000
7	400	KPO4	20	55	1455	1.094	1330	40	2000	1.387	1442	545	4000	3317	**1603**	**1739**	**558**	**100**	4000
8	400	KPO4	22	55	1600	1.094	1463	40	2200	1.387	1586	200	4000	3249	**1801**	**1953**	**146**	**100**	4000
9	400	Na citrate	16	55	1164	1.094	1064	40	1600	1.278	1252	1236	4000	3552	**1198**	**1410**	**1292**	**100**	4000
10	400	Na citrate	18	55	1309	1.094	1197	40	1800	1.278	1408	891	4000	3496	**1369**	**1612**	**919**	**100**	4000
11	400	Na citrate	20	55	1455	1.094	1330	40	2000	1.278	1565	545	4000	3440	**1546**	**1820**	**534**	**100**	4000
12	400	Na citrate	22	55	1600	1.094	1463	40	2200	1.278	1721	200	4000	3384	**1729**	**2035**	**136**	**100**	4000
13	400	NaPO4	16	55	1164	1.094	1064	29	2207	1.275	1731	629	4000	3424	**1243**	**2022**	**635**	**100**	4000
14	400	NaPO4	18	55	1309	1.094	1197	29	2483	1.275	1947	208	4000	3352	**1428**	**2324**	**148**	**100**	4000
15	400	NaSO4	16	55	1164	1.094	1064	29	2207	1.293	1707	629	4000	3400	**1251**	**2008**	**641**	**100**	4000
16	400	NaSO4	18	55	1309	1.094	1197	29	2483	1.293	1920	208	4000	3325	**1440**	**2310**	**150**	**100**	4000
17	400	NH4PO4	16	55	1164	1.094	1064	40	1600	1.241	1289	1236	4000	3589	**1185**	**1437**	**1278**	**100**	4000
18	400	NH4PO4	18	55	1309	1.094	1197	40	1800	1.241	1450	891	4000	3538	**1353**	**1640**	**907**	**100**	4000
19	400	NH4PO4	20	55	1455	1.094	1330	40	2000	1.241	1612	545	4000	3487	**1525**	**1849**	**526**	**100**	4000
20	400	NH4PO4	22	55	1600	1.094	1463	40	2200	1.241	1773	200	4000	3435	**1703**	**2064**	**133**	**100**	4000
21	400	NH4SO4	16	55	1164	1.094	1064	40	1600	1.230	1301	1236	4000	3601	**1182**	**1445**	**1273**	**100**	4000
22	400	NH4SO4	18	55	1309	1.094	1197	40	1800	1.230	1463	891	4000	3551	**1348**	**1648**	**904**	**100**	4000
23	400	NH4SO4	20	55	1455	1.094	1330	40	2000	1.230	1626	545	4000	3501	**1519**	**1858**	**523**	**100**	4000
24	400	NH4SO4	22	55	1600	1.094	1463	40	2200	1.230	1789	200	4000	3451	**1695**	**2073**	**132**	**100**	4000
25	1000	K citrate	14	55	1018	1.094	931	40	1400	1.289	1086	1582	4000	3599	**1035**	**1207**	**1658**	**100**	4000
26	1000	K citrate	16	55	1164	1.094	1064	40	1600	1.289	1241	1236	4000	3541	**1201**	**1402**	**1297**	**100**	4000
27	1000	K citrate	18	55	1309	1.094	1197	40	1800	1.289	1396	891	4000	3484	**1374**	**1603**	**923**	**100**	4000
28	1000	K citrate	20	55	1455	1.094	1330	40	2000	1.289	1552	545	4000	3427	**1552**	**1811**	**537**	**100**	4000
29	1000	K citrate	22	55	1600	1.094	1463	40	2200	1.289	1707	200	4000	3369	**1736**	**2026**	**137**	**100**	4000
30	1000	KPO4	14	55	1018	1.094	931	40	1400	1.387	1009	1582	4000	3522	**1057**	**1146**	**1697**	**100**	4000
31	1000	KPO4	16	55	1164	1.094	1064	40	1600	1.387	1154	1236	4000	3454	**1232**	**1336**	**1332**	**100**	4000
32	1000	KPO4	18	55	1309	1.094	1197	40	1800	1.387	1298	891	4000	3385	**1414**	**1533**	**953**	**100**	4000

Table 56. Aqueous two-phase systems made using the liquid-handling robot. Columns 2-4 show characteristic of ATPS: PEG molecular weight, salt type, concentrations. In columns 5-8 parameters of PEG stock solutions and in columns 9-12 the parameters of salt stock solution to make 4g of ATPS, are presented. In columns 16-19 volumes of PEG stock solutions, salt stock solution, water and a substrate stock solution to make 4ml of ATPS. These volumes were used to make ATPS by the robot. Page 1 of 3.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
No	**PEG type**	**Salt type**	**Concentration**	**PEG**				**Salt**				**Water [g,µl]**	**Total weight [mg]**	**Total volume [µl]**	**PEG volume [µl]**	**Salt volume [µl]**	**Water volume [µl]**	**Sample volume [ul]**	**Total volume [µl]**
				%	**M [mg]**	**Density**	**V [µl]**	**%**	**M [mg]**	**Density**	**V [µl]**								
33	1000	KPO4	20	55	1455	1.094	1330	40	2000	1.387	1442	545	4000	3317	**1603**	**1739**	**558**	**100**	4000
34	1000	KPO4	22	55	1600	1.094	1463	40	2200	1.387	1586	200	4000	3249	**1801**	**1953**	**146**	**100**	4000
35	1000	Na citrate	14	55	1018	1.094	931	40	1400	1.278	1095	1582	4000	3608	**1032**	**1214**	**1654**	**100**	4000
36	1000	Na citrate	16	55	1164	1.094	1064	40	1600	1.278	1252	1236	4000	3552	**1198**	**1410**	**1292**	**100**	4000
37	1000	Na citrate	18	55	1309	1.094	1197	40	1800	1.278	1408	891	4000	3496	**1369**	**1612**	**919**	**100**	4000
38	1000	Na citrate	20	55	1455	1.094	1330	40	2000	1.278	1565	545	4000	3440	**1546**	**1820**	**534**	**100**	4000
39	1000	Na citrate	22	55	1600	1.094	1463	40	2200	1.278	1721	200	4000	3384	**1729**	**2035**	**136**	**100**	4000
40	1000	NaPO4	14	55	1018	1.094	931	29	1931	1.275	1515	1051	4000	3496	**1065**	**1733**	**1102**	**100**	4000
41	1000	NaPO4	16	55	1164	1.094	1064	29	2207	1.275	1731	629	4000	3424	**1243**	**2022**	**635**	**100**	4000
42	1000	NaPO4	18	55	1309	1.094	1197	29	2483	1.275	1947	208	4000	3352	**1428**	**2324**	**148**	**100**	4000
43	1000	NaSO4	14	55	1018	1.094	931	29	1931	1.293	1493	1051	4000	3475	**1071**	**1719**	**1110**	**100**	4000
44	1000	NaSO4	16	55	1164	1.094	1064	29	2207	1.293	1707	629	4000	3400	**1251**	**2008**	**641**	**100**	4000
45	1000	NaSO4	18	55	1309	1.094	1197	29	2483	1.293	1920	208	4000	3325	**1440**	**2310**	**150**	**100**	4000
46	1000	NH4PO4	14	55	1018	1.094	931	40	1400	1.241	1128	1582	4000	3641	**1023**	**1239**	**1638**	**100**	4000
47	1000	NH4PO4	16	55	1164	1.094	1064	40	1600	1.241	1289	1236	4000	3589	**1185**	**1437**	**1278**	**100**	4000
48	1000	NH4PO4	18	55	1309	1.094	1197	40	1800	1.241	1450	891	4000	3538	**1353**	**1640**	**907**	**100**	4000
49	1000	NH4PO4	20	55	1455	1.094	1330	40	2000	1.241	1612	545	4000	3487	**1525**	**1849**	**526**	**100**	4000
50	1000	NH4PO4	22	55	1600	1.094	1463	40	2200	1.241	1773	200	4000	3435	**1703**	**2064**	**133**	**100**	4000
51	1000	NH4SO4	14	55	1018	1.094	931	40	1400	1.230	1138	1582	4000	3651	**1020**	**1247**	**1633**	**100**	4000
52	1000	NH4SO4	16	55	1164	1.094	1064	40	1600	1.230	1301	1236	4000	3601	**1182**	**1445**	**1273**	**100**	4000
53	1000	NH4SO4	18	55	1309	1.094	1197	40	1800	1.230	1463	891	4000	3551	**1348**	**1648**	**904**	**100**	4000
54	1000	NH4SO4	20	55	1455	1.094	1330	40	2000	1.230	1626	545	4000	3501	**1519**	**1858**	**523**	**100**	4000
55	1000	NH4SO4	22	55	1600	1.094	1463	40	2200	1.230	1789	200	4000	3451	**1695**	**2073**	**132**	**100**	4000
56	3350	K citrate	12	35	1371	1.060	1294	40	1200	1.289	931	1429	4000	3653	**1417**	**1019**	**1464**	**100**	4000
57	3350	K citrate	14	35	1600	1.060	1509	40	1400	1.289	1086	1000	4000	3596	**1679**	**1208**	**1012**	**100**	4000
58	3350	K citrate	16	35	1829	1.060	1725	40	1600	1.289	1241	571	4000	3538	**1950**	**1403**	**546**	**100**	4000
59	3350	K citrate	18	35	2057	1.060	1941	40	1800	1.289	1396	143	4000	3480	**2231**	**1605**	**64**	**100**	4000
60	3350	KPO4	12	35	1371	1.060	1294	40	1200	1.387	865	1429	4000	3588	**1443**	**965**	**1493**	**100**	4000
61	3350	KPO4	14	35	1600	1.060	1509	40	1400	1.387	1009	1000	4000	3519	**1716**	**1147**	**1037**	**100**	4000
62	3350	KPO4	16	35	1829	1.060	1725	40	1600	1.387	1154	571	4000	3450	**2000**	**1337**	**563**	**100**	4000
63	3350	KPO4	18	35	2057	1.060	1941	40	1800	1.387	1298	143	4000	3381	**2296**	**1535**	**69**	**100**	4000
64	3350	Na citrate	12	35	1371	1.060	1294	40	1200	1.278	939	1429	4000	3661	**1413**	**1026**	**1461**	**100**	4000
65	3350	Na citrate	14	35	1600	1.060	1509	40	1400	1.278	1095	1000	4000	3605	**1675**	**1216**	**1010**	**100**	4000
66	3350	Na citrate	16	35	1829	1.060	1725	40	1600	1.278	1252	571	4000	3548	**1945**	**1411**	**544**	**100**	4000
67	3350	Na citrate	18	35	2057	1.060	1941	40	1800	1.278	1408	143	4000	3492	**2223**	**1613**	**64**	**100**	4000

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APPENDIX

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
No	**PEG type**	**Salt type**	**Concentration**	**PEG**				**Salt**				**Water [g,µl]**	**Total weight [mg]**	**Total volume [µl]**	**PEG volume [µl]**	**Salt volume [µl]**	**Water volume [µl]**	**Sample volume [ul]**	**Total volume [µl]**
				%	**M [mg]**	**Density**	**V [µl]**	**%**	**M [mg]**	**Density**	**V [µl]**								
68	3350	NaPO4	12	35	1371	1.060	1294	29	1655	1.275	1298	973	4000	3565	**1452**	**1456**	**992**	**100**	4000
69	3350	NaPO4	14	35	1600	1.060	1509	29	1931	1.275	1515	469	4000	3493	**1729**	**1734**	**437**	**100**	4000
70	3350	NaSO4	12	35	1371	1.060	1294	29	1655	1.293	1280	973	4000	3547	**1459**	**1443**	**998**	**100**	4000
71	3350	NaSO4	14	35	1600	1.060	1509	29	1931	1.293	1493	469	4000	3472	**1739**	**1721**	**440**	**100**	4000
72	3350	NH4PO4	12	35	1371	1.060	1294	40	1200	1.241	967	1429	4000	3689	**1403**	**1048**	**1449**	**100**	4000
73	3350	NH4PO4	14	35	1600	1.060	1509	40	1400	1.241	1128	1000	4000	3638	**1660**	**1241**	**1000**	**100**	4000
74	3350	NH4PO4	16	35	1829	1.060	1725	40	1600	1.241	1289	571	4000	3586	**1924**	**1438**	**537**	**100**	4000
75	3350	NH4PO4	18	35	2057	1.060	1941	40	1800	1.241	1450	143	4000	3534	**2197**	**1642**	**62**	**100**	4000
76	3350	NH4SO4	12	35	1371	1.060	1294	40	1200	1.230	976	1429	4000	3698	**1399**	**1055**	**1445**	**100**	4000
77	3350	NH4SO4	14	35	1600	1.060	1509	40	1400	1.230	1138	1000	4000	3648	**1655**	**1248**	**997**	**100**	4000
78	3350	NH4SO4	16	35	1829	1.060	1725	40	1600	1.230	1301	571	4000	3597	**1918**	**1446**	**535**	**100**	4000
79	3350	NH4SO4	18	35	2057	1.060	1941	40	1800	1.230	1463	143	4000	3547	**2189**	**1650**	**61**	**100**	4000
80	8000	K citrate	12	30	1600	1.052	1521	40	1200	1.289	931	1200	4000	3652	**1666**	**1020**	**1214**	**100**	4000
81	8000	K citrate	14	30	1867	1.052	1774	40	1400	1.289	1086	733	4000	3594	**1975**	**1209**	**716**	**100**	4000
82	8000	K citrate	16	30	2133	1.052	2028	40	1600	1.289	1241	267	4000	3536	**2294**	**1404**	**202**	**100**	4000
83	8000	KPO4	12	30	1600	1.052	1521	40	1200	1.387	865	1200	4000	3586	**1696**	**965**	**1239**	**100**	4000
84	8000	KPO4	14	30	1867	1.052	1774	40	1400	1.387	1009	733	4000	3517	**2018**	**1148**	**734**	**100**	4000
85	8000	KPO4	16	30	2133	1.052	2028	40	1600	1.387	1154	267	4000	3448	**2352**	**1338**	**209**	**100**	4000
86	8000	Na citrate	12	30	1600	1.052	1521	40	1200	1.278	939	1200	4000	3660	**1662**	**1026**	**1212**	**100**	4000
87	8000	Na citrate	14	30	1867	1.052	1774	40	1400	1.278	1095	733	4000	3603	**1970**	**1216**	**714**	**100**	4000
88	8000	Na citrate	16	30	2133	1.052	2028	40	1600	1.278	1252	267	4000	3547	**2287**	**1412**	**201**	**100**	4000
89	8000	NaPO4	12	30	1600	1.052	1521	29	1655	1.275	1298	745	4000	3564	**1707**	**1457**	**736**	**100**	4000
90	8000	NaPO4	14	30	1867	1.052	1774	29	1931	1.275	1515	202	4000	3491	**2033**	**1735**	**132**	**100**	4000
91	8000	NaSO4	12	30	1600	1.052	1521	29	1655	1.293	1280	745	4000	3546	**1716**	**1444**	**740**	**100**	4000
92	8000	NaSO4	14	30	1867	1.052	1774	29	1931	1.293	1493	202	4000	3470	**2045**	**1721**	**133**	**100**	4000
93	8000	NH4PO4	12	30	1600	1.052	1521	40	1200	1.241	967	1200	4000	3688	**1650**	**1049**	**1202**	**100**	4000
94	8000	NH4PO4	14	30	1867	1.052	1774	40	1400	1.241	1128	733	4000	3636	**1952**	**1241**	**707**	**100**	4000
95	8000	NH4PO4	16	30	2133	1.052	2028	40	1600	1.241	1289	267	4000	3584	**2263**	**1439**	**198**	**100**	4000
96	8000	NH4SO4	12	30	1600	1.052	1521	40	1200	1.230	976	1200	4000	3697	**1646**	**1056**	**1199**	**100**	4000
97	8000	NH4SO4	14	30	1867	1.052	1774	40	1400	1.230	1138	733	4000	3646	**1947**	**1249**	**705**	**100**	4000
98	8000	NH4SO4	16	30	2133	1.052	2028	40	1600	1.230	1301	267	4000	3595	**2256**	**1447**	**197**	**100**	4000

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